THE TOXICITY OF HYDROXYLATED ALKYL-PHENANTHRENES TO EMBRYONIC FISH

by

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Abstract

Polycyclic aromatic hydrocarbons (PAH) are common aquatic contaminants found at industrially contaminated sites. Alkylated PAH have been identified as a component of oil that is chronically toxic to the early life stages of fish. These compounds are important target analytes in Natural Resource Damage Assessment following oil spills, and often the focus of remedial activities. However, the mechanisms of PAH and alkyl-PAH toxicity are not well understood.

The enzymatic metabolism of alkyl-PAH generates ring (OH-ring) and chain hydroxylated (OH-chain) derivatives, and has been associated with the increased prevalence of toxicity in early life stages (ELS) of fish. The role of PAH metabolism in toxicity remains unclear, and may involve the byproducts of metabolism such as reactive oxygen species (ROS), reactive intermediates, metabolites themselves, or a combination thereof. Using 1-methylphenanthrene (1MP) as a model alkyl-PAH, this research describes the relative toxicity of a suite of hydroxylated alkyl-PAH to the early life stages of fish, proposing an association between the formation of para-quinones and enhanced toxicity.

The results of this thesis demonstrate: (1) hydroxylated derivatives of 1MP differ in toxicity from their non-hydroxylated counterpart; (2) ring hydroxylated 1MP derivatives are more toxic than both chain-hydroxylated derivatives and 1MP itself; (3) the location of ring-hydroxylation can affect toxicity and (4) the octanol-water partition coefficient (K_{ow}) is a poor predictor of toxicity for hydroxylated APs derivatives.
Chapter 2 was co-authored by Dr. Peter Hodson, Dr. Toni Rantanen, Dr. Victor Snieckus, and Dr. Stephen Brown. Dr. Hodson contributed to experimental design, data analysis and interpretation, and edited all chapters. Dr. Rantanen also synthesized exposure chemicals. All co-authors also played an advisory role in the interpretation of toxicology results.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1MP</td>
<td>1-methylphenanthrene</td>
</tr>
<tr>
<td>1MP4</td>
<td>1-methyl-4-hydroxyphenanthrene</td>
</tr>
<tr>
<td>1MP7</td>
<td>1-methyl-7-hydroxyphenanthrene</td>
</tr>
<tr>
<td>1MP8</td>
<td>1-methyl-8-hydroxyphenanthrene</td>
</tr>
<tr>
<td>1MP9</td>
<td>1-methyl-9-hydroxyphenanthrene</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>Alkyl-PAH</td>
<td>Alkyl substituted polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>AP</td>
<td>Alkyl-phenanthrenes</td>
</tr>
<tr>
<td>AV</td>
<td>Atrio-ventricular</td>
</tr>
<tr>
<td>BaP</td>
<td>Benzo[α]pyrene</td>
</tr>
<tr>
<td>BH</td>
<td>Body hemorrhaging</td>
</tr>
<tr>
<td>βNF</td>
<td>β-naphthoflavone</td>
</tr>
<tr>
<td>BSD</td>
<td>Blue sac disease</td>
</tr>
<tr>
<td>CF</td>
<td>Craniofacial deformities</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CR</td>
<td>Circulation</td>
</tr>
<tr>
<td>CW</td>
<td>Water concentration</td>
</tr>
<tr>
<td>CW*</td>
<td>Critical concentration</td>
</tr>
<tr>
<td>CYP1A</td>
<td>Cytochrome P4501A enzyme</td>
</tr>
<tr>
<td>Cyp1a</td>
<td>Cytochrome P4501A gene</td>
</tr>
<tr>
<td>CYP450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DMBA</td>
<td>Dimethylbenz(α)anthracene</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpf</td>
<td>Days post fertilization</td>
</tr>
<tr>
<td>EC</td>
<td>Environment Canada</td>
</tr>
<tr>
<td>EC50</td>
<td>Median effective concentration</td>
</tr>
<tr>
<td>ELS</td>
<td>Early life stages</td>
</tr>
<tr>
<td>EROD</td>
<td>Ethoxyresorufin O-deethylase</td>
</tr>
<tr>
<td>ERS</td>
<td>Embryo rearing solution</td>
</tr>
<tr>
<td>ET50</td>
<td>Median effective time</td>
</tr>
<tr>
<td>FR</td>
<td>Fin rot</td>
</tr>
<tr>
<td>GJIC</td>
<td>Gap Junction Intercellular Communication</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>IEQ</td>
<td>Induction equivalents</td>
</tr>
<tr>
<td>IH</td>
<td>Improper hatch</td>
</tr>
<tr>
<td>Kow</td>
<td>Octanol-water partition coefficient</td>
</tr>
<tr>
<td>LC50</td>
<td>Median lethal concentration</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
</tbody>
</table>
MS  Mass spectroscopy
MS-222 Tricaine methane sulfonate
·OH  Hydroxyl radicals
O₂·- Superoxide radicals
OE  Ocular edema
OH-chain Chain hydroxylated
OH-MP 1-(hydroxymethyl)phenanthrene
OH-PAH Hydroxyl-PAH
OH-ring Ring hydroxylated
PAH  Polycyclic aromatic hydrocarbon
PCB  Polychlorinated biphenyl
PDA Fluorescence photodiode array
PE  Pericardial edema
PHH  Planar halogenated hydrocarbons
pKa Acid dissociation constant
QSAR Quantitative structure activity relationship
Retene 7-isopropyl-1-methylphenanthrene
ROS  Reactive oxygen species
SD  body axis/spinal deformities
SI  Severity index
sih  Silent heart mutant
TCDD 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD)
TEFs Toxic equivalency factors
TEQs Toxic equivalents
TH  Tube heart
TLM  Target lipid model
tPAH Total polycyclic aromatic hydrocarbon
TU Toxic units
TUₜₜ  Toxic potential in water
U.S.EPA United States Environmental Protection Agency
UV  Ultraviolet radiation
XRE  Xenobiotic response element
YE  Yolk sac edema
Chapter 1

General Introduction and Literature Review

1.1 Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic Aromatic hydrocarbons (PAHs) are a broad class of lipophilic environmental contaminants that are structurally distinguished by the presence of two or more fused aromatic rings. Typically, members of this chemical family are hydrophobic, semi-volatile, fluorophores, and found in air, water, and sediment (Dabestani & Ivanov, 1999). They are intermediates in the production of industrial products such as plastics and plasticizers, pigments and dyes, and pesticides (Bulder, et al., 2006). There are over 100 unique PAH based on ring structure alone, with consideration of substituted PAH such as alkyl and hydroxyl derivatives further expanding the breadth of this chemical group. These compounds can be divided into subclasses based on a variety of factors, including: number of rings, degree of substitution, identity of substituents, potency for a particular receptor, and physical and chemical characteristics.

1.1.1 PAHs in the Aquatic Environment

Polycyclic Aromatic hydrocarbons are present in the environment as complex mixtures, originating from petrogenic, biogenic, and pyrogenic sources. Natural sources of PAH include oil seeps, forest fires, and volcanoes, while anthropogenic inputs can include the burning of fossil fuels, production of coke and charcoal, metal smelting, petroleum refining, and petroleum spills (Rand, et al., 1995). Generally, the main concern
in aquatic ecosystems is the introduction of PAH originating from industrial effluents and petroleum spills. Sediment concentrations of a single PAH (retene) can reach 1600μg/g dry weight at sites receiving effluents from pulp and paper mills (Leppanen & Oikari, 1999). These measured PAH concentrations are important in Natural Resource Damage Assessment following oil spills or effluent release, and are often the focus of remedial activities.

Recently, alkyl substituted PAH (alkyl-PAH) have been identified as constituents of particular interest in petroleum contaminated sites. The use of effects-driven fractionation implicates 3 to 5-ring alkyl-PAH as the specific components in crude oil that are chronically toxic to the early life stages (ELS) of fish (Khan, 2007; Hodson, et al., 2007). Alkyl-PAH found in petroleum are more abundant and persistent than their non-alkylated homologue (Barron & Holder, 2003). Alkyl substitution increases molecular weight, increases lipophilicity, reduces volatility, and decreases hydrophilicity. Additionally, some alkyl-PAH such as 7-isopropyl-1-methylphenanthrene (retene), can be up to ten times more toxic than their un-substituted counterparts (Turcotte, et al., 2011). While typically grouped to simplify complex environmental scenarios, many individual PAH exhibit differential toxicity during embryonic and early larval development (Incardona, et al., 2004).

Although the cause of this difference in toxicity remains under investigation, it is believed to relate to the potency of these compounds for the aryl hydrocarbon receptor (AhR). While the majority of PAH are predicted to be type 1 narcotics, causing toxicity
via a non-specific mode of action (Dabestani & Ivanov, 1999), alkyl-PAH are among the PAH that induce cytochrome P4501A (CYP1A) and are metabolized into hydroxylated derivatives (Di Giulio, et al., 1995). It remains unclear whether the potential role of metabolism in alkyl-PAH toxicity is attributed to the resulting metabolites or to the byproducts of metabolism.

1.2 PAH: Mechanisms of Toxicity

PAHs are a broad class of compounds with some constituents demonstrating unique mechanisms of toxicity, as described by Billiard, et al. (2008). There is evidence for at least six different models of toxicity for PAH. Generally, toxicity can be classified as non-receptor mediated narcosis, indirect AhR mediated toxicity, indirect CYP1A mediated toxicity, direct receptor mediated disruption of embryonic cardiac development and function, photo-toxicity, and carcinogenicity.

1.2.1 Non-receptor Mediated: Narcosis

The term “narcotic” generally refers to any chemical agent that causes an anesthetic-like effect. Narcotics are believed to dissolve in the lipid bi-layer, interfering with membrane function. This mode of toxicity is believed to be a non-specific, reversible effect, which depends on the partitioning of a hydrophobic chemical into cell membranes. This suggests that the potency of narcotics is entirely dependent on their ability to bio-concentrate in lipids, as indicated by their octanol-water partition coefficient ($K_{ow}$), a concept further discussed in section 1.4.3 (Incardona, et al., 2006).
There are two categories of narcotics: inert or non-polar narcotics that do not interact with specific receptors are classified as type I, while less inert or polar narcotics without specific receptor interactions are classified as type II or polar narcotics (Verhaar, et al., 1992). Most PAH are predicted to act via narcosis. Particularly, two to four, and some five ring PAHs with no functional groups, are believed to be type I narcotics.

Similar to most PAH, general anesthetics were also believed to act via narcosis. Given that a wide range of structurally dissimilar compounds possessed anesthetic activity (Campagna, et al., 2003), and the strong relationship observed between their potency and their oil (Meyer, 1899; Overton, 1901) or lipid solubility (Janoff, et al., 1981), it was postulated that volatile anesthetics act non-specifically on hydrophobic lipid components of cells. Despite the elegance of this theory, the absence of strong evidence in support of narcosis as reviewed by Campagna, et al.(2003), and the identification of specific protein binding sites for anesthetics as reviewed by Franks (2006), has resulted in the rejection of narcosis as the mode of action for inhaled anesthetics.

Anesthetics are now believed to be much more selective than previously anticipated, and a group of ligand-gated ion-channels are considered the most plausible targets of anesthetic action as reviewed by Yamakura, et al., (2001). However, because ion channels function within lipid membranes, it is difficult to distinguish whether the observed anesthetics effects are caused indirectly due to loss of membrane integrity or by direct binding.
More recently, PAH have also been demonstrated to be more selective, with both direct and indirect receptor mediated effects. For example, phenanthrene which was predicted to act via type I narcosis, is suspected of having unique molecular targets resulting in the blockage of cardiac ion channels (Incardona, et al., 2004). It is possible that many chemicals, whose mode of action is typically generalized as narcosis, have membrane-targeted mechanisms. In this scenario, the capacity of $K_{ow}$ as a predictor of toxicity may relate more accurately to its description of exposure kinetics, not toxicity.

1.2.2 Indirect Receptor Mediated: AhR Mediated Dioxin-like Toxicity

Not all PAH are toxic by narcosis. Some non-chlorinated PAHs, particularly alkyl-PAH such as retene, can mimic the embryotoxic effects that are typically associated with planar halogenated hydrocarbons (PHHs) such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) (Billiard, et al., 1999). For such compounds, toxicity is receptor mediated; correlating to the compound’s binding affinity for the aryl hydrocarbon receptor (AhR) (Billiard, et al., 2002).

The AhR is a ligand-activated transcription factor that exists as a cytosolic complex in the absence of inducers. Ligand binding triggers translocation of the AhR to the nucleus, where its interaction with the xenobiotic response element (XRE) and up-regulates the transcription of the cyp1a gene battery, as illustrated in Figure 1-1 (Mimura & Fujii-Kuriyama, 2003). The binding affinity of PAHs for the AhR is predictive of their potency for CYP1A induction (Billiard, et al., 2002).
Cytochrome P4501A or CYP1A, is a member of a family of heme containing enzymes, collectively referred to as cytochrome P450 (CYP450). These enzymes catalyze multiple hydroxylation, epoxidation, de-alkylation, de-amination, sulfoxidation, and de-sulfuration reactions in the cell (Di Giulio, et al., 1995). The CYP1A isoform is the main hydrocarbon inducible form of cytochrome P450 in fish and other vertebrates (Di Giulio, et al., 1995). However, CYP1A induction is difficult to measure and typically is not quantified directly. Ethoxyresorufin O-deethylase (EROD) measures the enzymatic action of CYP1A proteins in catalyzing the de-ethylation of ethoxyresorufin to resorufin. The activity of EROD is often used to estimate CYP1A activity in the liver tissue.

**Figure 1-1** Induction of cytochrome P4501A (CYP1A) enzymes by the ligand activated aryl hydrocarbon receptor (AhR) complex. The activated AhR complex moves into the nucleus, dimerizes with the AhR nuclear translocator (Arnt) protein, and interacts with the xenobiotic response elements (XRE) leading to the transcription of the *cyp1a* gene battery, and the up-regulation of CYP1A enzymes.
Mixtures of PAHs with similar potency for the AhR can cause additive induction of CYP1A enzymes, while PAH mixtures composed of weak and strong AhR inducers can generate 800-900% greater CYP1A induction than expected based on additive models (Basu, et al., 2001). To date, cardiovascular endothelium is considered the most sensitive vertebrate tissue type affected by AhR dependent CYP1A induction, and the cardiovascular system appears to be the primary target of PAH toxicity to the ELS of fish (Incardona, et al., 2004). Expression of the cyp1a gene battery at an inappropriate time during embryonic development can exert unwanted biologic effects through the CYP1A metabolism of endogenous substrates such as arachidonic acid, estrogens, bilirubin, and melatonin, and the over expression of this gene assemblage is associated with a suite of toxic effects including: pericardial and yolk sac edema, craniofacial and spinal deformities, and cardiac arrhythmia (Rifkind, 2006).

Some PAH components of heavy fuels such as phenanthrene do not bind to the AhR or induce enhanced CYP1A activity. However, in the presence of a CYP1A inducer such as β-naphthoflavone (βNF), phenanthrene metabolism, the excretion rate of its metabolites, and its toxicity are enhanced. In the absence of inducers, toxicity does not decrease with CYP1A inhibition, phenanthrene is therefore said to become “activated” by CYP1A inducers (Hawkins, et al., 2002). Also, the addition of alkyl groups to these PAH seems to shift the mode of toxicity to AhR mediated dioxin-like toxicity. These PAH appear to become AhR agonists upon alkylation, and exhibit greater toxicity (Turcotte, et al., 2011).
1.2.3 Indirect Receptor Mediated: CYP1A Mediated Toxicity

Prolonged CYP1A activation through continuous exposure to PAHs such as retene can also enhance toxicity. Cytochrome P450 hydroxylation reactions introduce one atom of oxygen to an organic substrate. This catalysis reaction can also produce unwanted byproducts in the form of reactive oxygen species (ROS). These byproducts can include hydroxyl radicals (·OH), superoxide radicals (O₂·⁻), and hydrogen peroxide (H₂O₂). An abundance of ROS can lead to oxidative stress, causing lipid membrane damage, and protein and polysaccharide degradation (Turrens, 2003).

Oxidative stress has been implicated as a factor in many morphological abnormalities associated with oil exposure, including: edema, hemorrhaging, and craniofacial deformities, which may be indicative of membrane damage, circulatory failure, and impaired development, respectively. Fish exposed to retene have a higher prevalence of morphological abnormalities and lower tissue concentrations of antioxidants such as vitamin E and glutathione. Co-exposure of embryos to retene and vitamin E lowers the prevalence of these abnormalities without affecting CYP1A levels (Bauder, et al., 2005).

The metabolism of alkyl-PAHs generates ring (phenols) and chain hydroxylated (benzylic alcohols) derivatives (Tabash, 2002). There is some indication that these derivatives may cause further toxicity either through the generation of additional ROS by undergoing further metabolism, or due to a direct receptor mediated affect.

In the case of alkyl-PAHs such as retene, CYP450 hydroxylation reactions involving the alkyl group’s α carbon have been implicated in the formation of reactive
intermediates. Additionally, sulfonation of hydroxyl metabolites by the sulfotransferase enzyme generates leaving groups that form carbocations upon cleavage. These carbocations can then form adducts with cellular components such as DNA, leading to genotoxicity (Di Giulio, et al., 1995).

Investigations of petroleum-derived PAHs such as alkyl-phenanthrenes (APs), link metabolism and the prevalence of toxicity in ELS of fish (Turcotte, et al., 2011). These findings implicate a relationship between structure and toxicity, and highlight the key role metabolism may play in the enhanced toxicity of some PAHs.

1.2.4 Direct Receptor Mediated: Disruption of Embryonic Cardiac Function

Typically, PAHs are believed to act non-specifically through narcosis, or indirectly through activation of the AhR pathway leading to CYP1A induction. However, recent evidence seems to suggest an alternative AhR mediated mechanism of toxicity independent of CYP1A, which targets the cardiovascular system, specifically producing the developmental defects associated with three-ring PAHs (Scott, et al., 2011). Such findings further challenge our understanding of the mechanisms of PAH toxicity.

Some three-ring PAH may also have unique molecular targets that result in blockage of cardiac ion channels leading to a 2:1 atrio-ventricular conduction block, a particular type of arrhythmia (Incardona, et al., 2004). Embryos exposed to non-alkylated three-ring PAHs, particularly phenanthrene, exhibit developmental abnormalities caused by the genetic disruption of cardiac function.
The zebrafish (*Danio rerio*) mutant silent heart (*sih*) does not develop a heartbeat. Targeted genetic disruption of heart development using *sih* antisense morpholinos results in a suite of developmental defects similar to those induced by three-ring PAHs. These defects included mild pericardial edema, dorsal curvature of the body axis, reduced eye and jaw growths, bradycardia and arrhythmias characteristic of a 2:1 AV conduction block, where the atrium beats twice for every ventricular contraction (Incardona, et al., 2004).

1.2.5 Photo-toxicity

Many laboratory studies take place under fluorescent lights, with little UV exposure. Ultraviolet radiation (UV) is a component of sunlight and can be absorbed by some components of heavy oil, including PAHs. Aquatic environments are subject to both UV-B (280 to 320 nm) and UV-A (320 to 400 nm) exposure (Barron, et al., 2003). Some PAHs, such as acridine, anthracene, benzanthrone, benzo(α)anthracene, pyrene, and benzo(α)pyrene, demonstrate photo-induced toxicity (Oris & Giesy, 1987). Phototoxicity results from the absorption of UV radiation by the conjugated bonds present in some PAH. The transfer of this absorbed energy to a ground state oxygen molecule can result in the production of ROS, thus enhancing toxicity. Also, the absorption of UV energy can cause some PAHs to form reactive intermediates that interact with DNA forming adducts and leading to genotoxicity (Pelletier, et al., 1997).

The presence of UV-B light enhances the ability of PAHs such as retene to induce CYP1A in some embryonic and larval fish, and can increase the toxicity of formerly sub-
lethal concentrations to lethal levels (Vehnianen, et al, 2003). Additionally, many PAHs of relatively low toxicity are more toxic to embryonic and larval stages of fish in the presence of UV radiation. Anthracene can be 190-1800 times more lethal with concurrent exposure to simulated sunlight, with affected fish exhibiting signs of irritation and hypoxia (Oris et al., 1985).

Simultaneous exposure of whitefish larvae (*Coregonus lavaretus* s.l.) to retene and UV-B causes enhanced toxicity in the form of additional behavioral abnormalities such as uncontrolled spiral swimming, hypoxia as suggested by a tendency to swim to the water-air interface, severe skin damage accompanied by an increase in mucus cells, and hepatic lesions indicative of hepatotoxicity (Häkkinen, et al., 2003).

Photo-enhanced toxicity is only observed if oil or PAH are present in larval tissue. Studies conducted on Japanese medaka (*Oryzias latipes*) using alkyl-PAH components of fuel oil such as alkyl-anthracenes indicate that the PAH photoproducts (photo-degraded PAH) alone are not toxic to fish (Turcotte, et al., 2008). However, toxicity increased with increasing UV treatment and with increasing total PAH concentration in tissue. Co-exposure to UV radiation can lead to an 18-450 fold increase in oil toxicity when compared with values measured under normal laboratory test conditions with fluorescent lighting (Barron, et al., 2003). The photo-toxic potential of petroleum products is dependent on the composition and concentrations of the photo-toxic PAHs present.
1.2.6 Carcinogenesis

Some PAH can act as pro-carcinogens and tumor initiators, mutagens, and tumor promoters (Pitot & Yvonne, 2001). For example, BaP and 7,12-dimethylbenz(α)anthracene (DMBA) are pro-carcinogens that can be metabolically activated by CYP450 enzymes to produce highly reactive epoxides, and subsequently diol-epoxide intermediates that like ROS interact with DNA to form adducts that can lead to tumor genesis (Shugart, 1995). Some alkyl-PAH are active mutagens, agents causing DNA damage. Metabolic studies focusing on substituted phenanthrenes indicate that the 9,10-dihydrodiol generated by CYP450 metabolism is a major metabolite of these alkyl-PAH. The presence of methyl substituents at or adjacent to the 9,10 position in these chemicals inhibits the formation of this diol (LaVoie, et al., 1981), and may be associated with mutagenicity (LaVoie, et al., 1981, 1983).

The mechanisms by which PAH can act as tumor promoters are not well understood. However, it has been suggested that PAH-induced alterations in mammalian intracellular Ca^{2+} levels may play a role in tumour promotion (Tannheimer, et al., 1997). Additionally, many PAH can interfere with Gap Junction Intercellular Communication (GJIC), which enables individual cells to communicate and maintain normal tissue structure. Inhibition of GJIC leads to uncontrolled cellular growth, which can result in tumor formation (Blaha, et al., 2002). Carcinogenesis is an important endpoint in risk assessments studies pertaining to human health. However, this endpoint is not nearly as relevant an indicator for aquatic ecosystem health, or that of fish populations, where recruitment decline is often the major concern.
1.3 PAH: Signs of Toxicity in Fish

Describing the signs of PAH toxicity can be exceptionally challenging. Numerous laboratory and field studies have illustrated the sub-lethal toxicity of PAH using various morphological, cellular, and subcellular endpoints including: arrested growth, hatching success, swimming ability, gonad size and reproductive success, presence of abnormalities, lesions, and DNA adducts. Much of the work following the Exxon Valdez oil spill documented hallmark signs of oil related toxic injury to the ELS of fish, which include pericardial and yolk sac edema, spinal deformities, circulation anomalies, fin rot, hemorrhaging, and craniofacial abnormalities, collectively referred to as blue sac disease.

1.3.1 Blue Sac Disease

Recognized since the early nineteen hundreds, blue sac disease (BSD) is a non-contagious irreversible condition first observed in salmonid sac fry, associated with physical and thermal shock, as well as excess ammonia and synthetic nitrogen compounds (Wolf, 1957). Blue sac disease has also been associated with CYP1A induction, ocular and yolk sac edema, hemorrhaging, and spinal and craniofacial deformities (Bauder, et al., 2005). These sub-lethal abnormalities can influence the survival potential of larvae by affecting their ability to flee from predators and obtain prey. Laboratory studies have also shown that exposure to AhR agonists, such as retene (Billiard, et al., 1999), TCDD (Hornung, et al., 1999), and coplanar polychlorinated
biphenyls (PCBs) (Walker, et al., 1996), is associated with an elevated prevalence of BSD in the early life stages of fish.

1.4 PAHs and the Estimation of Risk

Risk assessments attempt to identify concentrations at which pollutants cause irreversible harm to the environment, by making connections between some measure of exposure and various measures of damage. Contaminants such as PAH can exist in a variety of forms in aquatic environments, including: dissolved, bound to dissolved organic matter, adsorbed to suspended particulate matter, and associated with surface sediments or soils. The unique physiochemical properties of each PAH will dictate the extent of its interaction with each of these compartments, and resulting bioavailability to aquatic organisms. Generally, PAH have low solubility and a high affinity for organic carbon, causing them to preferentially bind with sediment or suspended sediment (Lyman, 1995).

The prevalence, movement, and identity of PAH have long been used as a metric of present and potential future resource damage following petroleum spills. Environmental risk assessment efforts following major spills, such as the Exxon Valdez (Carls, et al., 1999), the COSCO Busan (Incardona, et al., 2008), and the Deepwater Horizon oil spill (Diercks, et al., 2010) focus on PAH as constituents of particular interest. Unfortunately toxicity data are often unavailable for many PAH. While the importance of alkyl substitution and hydroxylation has been discussed in more recent literature, there is no clear consensus or standardized method for the use of this
information in damage assessment. Current risk assessment methods can be divided into four major approaches: the use of total PAH concentrations, the use of equivalency factors, use of octanol water partition coefficients, and toxic potential.

1.4.1 Total PAH (tPAH)

By far the crudest approach to assessing toxicity based on PAH concentrations is the use of total PAH (tPAH) concentrations. This approach does not discriminate among PAH with varying numbers of rings or substituents. Typically used in the initial steps of a risk assessment, tPAH data can indicate the presence and abundance of PAH at a particular site. The main criticism of this model’s capacity to estimate risk is its inability to differentiate among PAH, making comparison of effects concentrations difficult to impossible. It is well understood that the toxicity of a mixture of PAH depends not only on the concentrations but also the identity of PAH present. The use of tPAH values to assess toxicity assumes all PAH will have identical and additive affects. Using a numeric value for tPAH toxicity is essentially meaningless, as many PAH exhibit unique toxicity (Di Toro, et al., 2007).

1.4.2 Equivalency Factors

The use of equivalency factors decreases some of the uncertainty involved in site specific risk assessment by considering the relative toxic potency of different PAH. Equivalency factors are a weighted value system that accounts for some of the variation in the toxicity of different congeners of a particular chemical class or subclass, expressing the toxicity of less toxic compounds as fractions of the toxicity of the most toxic
congener. Typically, the most potent and well-characterized chemical of the class is selected as a reference for all remaining members to be compared to. This approach requires the toxic effects of all members of the class to be similar to those of the surrogate compound, and the toxic effects of their mixtures to be additive (Nisbet & LaGoy, 1992). Equivalency factors are used for risk characterization and management, typically as a tool for the identification and prioritization of areas of concern.

In the case of PAH, the toxic equivalency factors (TEFs) approach simplifies the toxic effects by focusing on carcinogenicity as the primary concern for risk assessment. Benzo[a]pyrene or BaP, a well characterized pro-carcinogen, is used as the reference compound as it is one of the most potent carcinogen in this chemical class. All remaining PAH are separated into two subclasses, carcinogenic and non-carcinogenic. The toxicity of all carcinogenic PAH is weighted against that of BaP which is assigned a TEF of one, and their corresponding TEFs expressed as fractions ranging from 0 to 1. If a PAH is non-carcinogenic it is assigned as value of zero (Nisbet & LaGoy, 1992). Variations of the equivalency factor method include the use of TEFs for dioxins, furans, and PCBs, and toxic equivalents (TEQs) for dioxins and dioxin-like compounds.

The chronic exposure of the ELS of fish to PAH is also a major concern in petroleum contaminated sites. Many chronically toxic PAH such as retene are not carcinogens. Potency data for non-cancer endpoints, such as acute, chronic, reproductive, developmental, and immune-toxicity, as well as receptor binding data can be used following a similar method. The induction equivalents (IEQ) approach allows for
equivalency factors to be applied to other endpoints such as enzyme induction (Parrott, et al., 1995).

Alkyl and non-alkyl PAH show a wide range of potencies for inducing CYP1A enzymes in fish. Modulating the activity of this family of enzymes has been correlated to marked changes in metabolism, excretion, and toxicity of some PAH (Hawkins, et al., 2002). Consequently, induction equivalency factors can be a very useful risk assessment tool in examining mixture toxicity. However, this approach is limited by the availability of mechanistic toxicity information, and the limited number of compounds tested within each PAH subclass. Variations in toxicity mechanisms within a class or subclass of PAH, as well as non-additive interactions, can further hinder the predictive ability of this risk assessment method.

1.4.3 Octanol Water Partition Coefficient (K_{ow})

Partitioning of PAH from the aquatic environment into organisms can be simplified by generalizing aquatic organisms as membrane delimited sacs containing water and lipid (Spacie, et al., 1995). Most PAH are expected to behave as type I narcotics, non-ionic organic chemicals with a non-specific mode of action that is believed to involve cell membrane phospholipids. The potency of narcotics depends entirely on the extent of their hydrophobicity, which allows for the prediction of expected effects concentrations using measures of compound hydrophobicity such as the octanol water partition coefficient (K_{ow}) (Di Toro, et al., 2000). As discussed in section 1.2.1, this parameter can be used as an index of toxicity because it predicts the kinetics of exposure
due to water: lipid partitioning. It describes the relationship between a compound’s solubility in n-octanol (a lipid surrogate) and its solubility in water, at equilibrium and at a specific temperature, defined as \[ \log K_{ow} = \log \left( \frac{[\text{Chemical}]_{\text{octanol}}}{[\text{Chemical}]_{\text{water}}} \right). \]

Current risk assessment models assume additive toxicity. Many quantitative structure activity models for narcotic toxicity rely on the relationship between toxicity parameters such as the median lethal concentration (LC50) and the octanol water partition coefficient or \( K_{ow} \) (Black, et al., 1983). The target lipid model (TLM) was developed to predict the toxicity of narcotic chemicals to aquatic organisms. It is based on the assumption that lipid is the target tissue for narcotics, and that mortality occurs once the chemical concentration in the target lipid reaches a species-specific threshold concentration. This model hypothesizes that octanol is an appropriate surrogate for the target lipid, and that the target lipid has the same physical and chemical properties in all organism. Approaches such as the TLM have accounted for much of the variation in existing toxicity data, successfully predicting both the acute and chronic toxicity of many PAHs, both in individual exposures and in mixtures, using their predicted \( K_{ow} \) values (McGrath & Di Toro, 2009).

Using \( K_{ow} \) intrinsically distinguishes between some PAH subclasses, such as alkyl and non-alkylated, and hydroxyl and non-hydroxylated homologues. However, there are limitations to the use of \( K_{ow} \) as a predictor of toxic effects. This parameter is not an accurate measure of hydrophobicity for ionizable compounds (polar organics). Also, experimental \( K_{ow} \) estimates are unavailable for many emerging contaminants, increasing
reliance on modeling software to predict $K_{ow}$ values. Because a wide array of PAH derivatives and metabolites share the same molecular weight and many structural characteristics, it can be difficult for computer simulations to differentiate among various congeners.

1.4.4 Toxic Potential and Solubility

Toxic potential integrates the toxicity of individual components of oil with their water solubility, allowing for a more environmentally relevant expression of their aquatic toxicity. Toxic units (TU) are usually used to compare the toxic potential of various compounds, where a toxic unit represents the ratio of the concentration of the compound in the water ($C_w$) column and the critical concentration ($C_{w*}$), or $TU = \frac{C_w}{C_{w*}}$. Critical concentration refers to the chemical concentration required to generate some endpoint, examples of which can include $LC_{50}$ and $EC_{50}$ values. The toxic potential of oil components ($TU_{w, max}$) such as PAH is defined as the toxicity of their saturated aqueous solution (i.e. at their solubility limit), a concentration that is predicted to also exert the maximum toxicity, $TU_{w, max} = \frac{C_{w_{max}}}{C_{w*}}$ (Di Toro, et al., 2007). Toxic units can be subsequently added to extrapolate mixture toxicity.

This method incorporates environmental factors such as salinity, temperature, and weathering. However, while this approach to assessing toxicity allows for the assessment of mixture toxicity in changing environments, it requires the toxicity of the chemicals in question to be very well understood. Consequently, this approach is not very useful in
examining the risk of PAH derivatives such as alkyl and hydroxyl-PAH (OH-PAH) whose toxicity at given aqueous concentrations is not well understood.

1.5 The Importance of Metabolism

Existing risk assessment methods for PAH, and alkyl-PAH in particular, are hindered by the unavailability of toxicity data for metabolites. It remains unclear whether alkyl-PAH toxicity is attributed directly to the alkyl-PAH, the generation of ROS as byproducts of the metabolic process, the metabolites themselves, or to some combination thereof. If toxicity was caused solely by the generation of ROS due to CYP1A metabolism, alkyl-PAH that exhibit higher rates of metabolism by CYP1A would exhibit the highest toxicity. However, while the potency for CYP1A induction and the rate of metabolism for alkyl-PAH follow the same trend, this pattern does not correspond with toxicity ranks (Tabash, 2002). This would in turn suggest that toxicity cannot be attributed to metabolic byproducts alone, leaving metabolites as the possible cause for the increased toxicity observed with some alkyl-PAH compared to their non-alkylated homologues.

The contrast between phenanthrene and retene, two well-studied three-ring PAH, well illustrates the differences in toxicity, and representation in monitoring schemes, between alkyl-PAH and their non-alkylated homologues, and further highlights the significance of understanding the role of metabolism in PAH toxicity. Phenanthrene is a characteristic byproduct of the combustion, and a component, of fossil fuels (Prahl & Carpenter, 1984). It is monitored by the United States Environmental Protection Agency
Retene, an alkyl-homologue of phenanthrene, and other alkyl-PAH are not specifically monitored by government agencies, though retene has begun to be incorporated into some monitoring schemes. Retene is a product of the incomplete combustion of wood and the biotransformation product of abietic acid, a wood resin, often found at industrially-contaminated sites and in pulp and paper mill effluents (Leppanen & Oikari, 1999). Chronic toxicity studies using Japanese medaka illustrate that retene is roughly ten times more toxic than phenanthrene (Turcotte, et al., 2011), and appears to be toxic via a different mechanism (Hawkins, et al., 2002).

Modulating CYP1A metabolism affects the toxicity and excretion of phenanthrene and retene in fish (Hawkins, et al., 2002). Retene binds to fish AhR (Billiard, et al., 2002), inducing CYP1A enzymes (Fragoso, et al., 1998). Its metabolites concentrate in bile, and do not accumulate in fish tissue (Hawkins, et al, 2002). In contrast, phenanthrene does not have a detectable binding affinity for fish AhR (Billiard, et al., 2002) nor does it induce CYP1A enzymes (Fent & Batscher, 2000). While metabolized by endogenous levels of CYP1A, its metabolism produces few metabolites in bile, and it accumulates in fish tissue to a greater extent than retene. Inducing CYP1A metabolism results in increased ELS toxicity in phenanthrene exposed fish, while CYP1A
inhibition increases toxicity and alters the abundance and persistence of specific retene metabolites (Hawkins, et al., 2002).

The ten-fold difference between retene and phenanthrene toxicity in fish is believed to be linked to specific stages of metabolism, or particular metabolites. The increase in retene toxicity associated with the accumulation of a subset of metabolites, coupled with the lack of direct correlation between the rate of substrate metabolism and toxicity, appears to point to particular metabolites as having a causal role in alkyl-PAH toxicity. However, there are insufficient toxicity data available to clarify the role of metabolism and metabolites in PAH toxicity.

1.6 Methyl-phenanthrene as a Model Alkyl-PAH

One approach to understanding the importance of alkyl-PAH metabolism, is to take a closer look at potential alkyl-PAH metabolites. The complex nature of alkyl-PAH metabolism, and the abundance of potential alkyl-PAH compounds, and their numerous metabolites complicates this task. While general PAH metabolism by CYP1A enzymes is well characterized, our knowledge regarding the resulting metabolites is very limited. 1-methylphenanthrene (1MP) is one of the simplest three-ring alkyl-PAH. It induces BSD and is among the least toxic alkyl-PAH, with similar toxicity to phenanthrene and 8-12 times lower toxicity than retene, a commonly used model compound for PAH and alkyl-PAH toxicity (Turcotte, et al., 2011).

Additionally, 1MP is one of a select few compounds whose metabolites as generated by fish CYP1A enzymes have been previously characterized (Figure 1-2).
Metabolism of alkyl-PAH by CYP1A generates two main groups of metabolites, ring-hydroxylated (phenols), and chain-hydroxylated (benzylic alcohols). High Pressure Liquid Chromatography (HPLC) with fluorescence photodiode array (PDA) and mass spectroscopy (MS) allows for the selective detection of these two isomer classes. Chain-hydroxylation is a major metabolic pathway for APs, and the relative proportion of ring and chain hydroxylated metabolites is unique to each AP of interest (Tabash, 2002).

Figure 1-2 In vitro formation of 1-methylphenanthrene (1MP) metabolites by cytochrome P450 enzymes: (M1-M5) are ring hydroxylated metabolites; (M6) is a chain hydroxylated metabolite; (M7+M8) are further oxidized metabolites; (P1) is 1MP; and (P2) is a phenanthrene recovery standard. Enzymatic reaction was quenched after 15mins. Chromatogram was collected with fluorescence detection (excitation 254nm/emission 370nm). Modified from Tabash (2002).

1.7 Thesis Objectives

Previous work suggests that AP toxicity is not caused solely by ROS, as the prevalence of toxicity is incongruent with the rate of AP metabolism and therefore ROS formation (Tabash, 2002). If AP toxicity is metabolite mediated, the prevalence of
toxicity may be linked to the formation of particular metabolite classes. While a link between the formation of chain-hydroxylated metabolites and toxicity has been postulated based on the potential for this derivative to form reactive carbocations in the cell, this theory has not been further investigated.

My working hypothesis is that AP toxicity is mediated by CYP1A metabolism and the formation of hydroxylated metabolites. Using 1MP as a reference compound, I investigated the chronic toxicity of four ring-hydroxylated 1MP derivatives, 1-methyl-4-hydroxyphenanthrene (1MP4), 1-methyl-7-hydroxyphenanthrene (1MP7), 1-methyl-8-hydroxyphenanthrene (1MP8), and 1-methyl-9-hydroxyphenanthrene (1MP9), and one (the only) chain-hydroxylated 1MP derivative, 1-(hydroxymethyl)phenanthrene (OH-MP). The following null hypotheses were tested: (1) there are no differences in toxicity between hydroxylated APs and their non-hydroxylated counterpart; (2) there are no differences in the toxicity between ring and chain-hydroxylated AP derivatives; (3) there are no differences among the toxicities of different ring-hydroxylated derivatives; and (4) there is no relationship between estimates of K_{ow} and the toxicity of hydroxylated APs.
1.8 References


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Chapter 2

Toxicity of Hydroxylated Alkyl-Phenanthrenes to the Early Life Stages of Japanese medaka (*Oryzias latipes*)
Abstract

Polycyclic aromatic hydrocarbons (PAH) are hydrophobic environmental contaminants with petrogenic, biogenic, and pyrogenic sources. Alkyl-PAH predominate in crude oils, are found in sediment downstream of pulp and paper mills, and can be more toxic than their non-alkylated homologues. The enzymatic metabolism of alkyl phenanthrenes (APs) generates ring (OH-ring) and chain hydroxylated (OH-chain) derivatives. While metabolism has been associated with an increased prevalence of toxicity in early life stages (ELS) of fish, it remains unclear whether this metabolic enhancement of toxicity can be attributed to the byproducts of metabolism such as reactive oxygen species (ROS) and reactive intermediates, or the metabolites themselves.

The main objective of this research was to estimate the potential role of hydroxylated alkyl-PAH derivatives in PAH metabolism and toxicity. This project assessed the relative toxicity of ring and chain hydroxylated 1-methylphenanthrenes to the ELS of Japanese medaka (Oryzias latipes), comparing them to one another and to their non-hydroxylated counterpart. Results indicate phenols are more toxic than benzylic alcohols, and some phenols are more than four times more toxic than their non-hydroxylated counterpart. This paper is the first to describe the relative toxicity of a suite of hydroxylated alkyl-PAH to the early life stages of fish, proposing an association between the preferential formation of para-quinones and enhanced toxicity.
2.1 Introduction

Polycyclic aromatic hydrocarbons (PAH) are a broad class of environmental contaminants, with some constituents demonstrating unique mechanisms of toxicity (Billiard, et al., 2008). Alkyl-PAH are the predominant form of PAH in crude oils (Wang, et al., 2003), more abundant and persistent than their non-alkylated homologues (Barron & Holder, 2003). Environmental risk assessment efforts following major oil spills, such as the Exxon Valdez (Carls, et al., 1999), the COSCO Busan (Incardona, et al., 2008), and the Deepwater Horizon (Diercks, et al., 2010), focus on PAH as important target analytes in remedial efforts. Specifically, 3 to 5-ring alkyl-PAH have been implicated as the principal agents of oil toxicity (Khan, 2007; Hodson, et al., 2007b), and may cause embryotoxicity, blue sac disease, and recruitment failure in fish spawning near oil contaminated shoals (Carls, et al., 1999).

Blue sac disease (BSD) is a non-contagious, irreversible suite of morphological abnormalities associated with chemical, physical, and thermal shock (Wolf, 1957). Signs of BSD due to dioxin-like chemicals include ocular and yolk sac edema, hemorrhaging, circulatory abnormalities, and spinal and craniofacial deformities. Exposure to some alkyl-PAH (Bauder, et al., 2005), 2,3,7,8-tetrachlorodibenzodioxin TCDD (Hornung, et al., 1999), and coplanar polychlorinated biphenyls (PCBs) (Walker, et al., 1996), has been linked to the elevated prevalence of BSD in the early life stages of fish. These sub-lethal abnormalities can influence the survival potential of larvae by affecting their ability to flee from predators and attain prey (Carls, et al., 1999).
Alkylated PAH such as 7-isopropyl-1-methylphenanthrene (retene), induce BSD and are up to ten times more toxic than their non-alkylated counterparts (Turcotte, et al., 2011). The cause of this difference in toxicity may relate to the potency of these compounds for the aryl hydrocarbon receptor (AhR), a ligand activated transcription factor. While the majority of PAH are predicted to be type 1 narcotics, causing toxicity via a non-specific mode of action (Dabestani & Ivanov, 1999), some PAH interact with AhR, inducing cytochrome P4501A (CYP1A). This heme containing enzyme generates hydroxylated metabolites by catalyzing hydroxylation reactions (Di Giulio, et al., 1995).

It is unclear whether alkyl-PAH toxicity is caused directly by the alkyl-PAH, indirectly by metabolic byproducts such as reactive oxygen species (ROS), directly by metabolites themselves, or some combination thereof. Risk assessments for alkyl-PAH are hindered by the limited toxicity data for these compounds and their metabolites.

Oxidative stress has been implicated as a factor in many morphological abnormalities associated with oil exposure, which may be indicative of membrane damage, circulatory failure, and impaired development. Fish exposed to alkyl-PAH have a higher prevalence of morphological abnormalities and lower tissue concentrations of antioxidants such as vitamin E and glutathione. Co-exposure of embryos to retene and vitamin E lowers the prevalence of these abnormalities without affecting CYP1A levels (Bauder, et al., 2005). However, the rate of AP metabolism does not follow the same rank order as the prevalence of toxicity, which implies toxicity is not due solely to the overproduction of metabolic byproducts such as ROS (Tabash, 2002). Additionally,
modulating the activity of CYP1A enzymes has been correlated to marked changes in metabolism, excretion, and toxicity of some PAH.

Inducing CYP1A metabolism results in increased ELS toxicity for alkyl-PAH, and alters the abundance and persistence of specific retene metabolites (Hawkins, et al., 2002). Partial inhibition of CYP1A catalyzed oxygenation reactions increases toxicity along with the abundance of less polar metabolites and parent retene in tissue, while further inhibition of CYP1A metabolism eliminates retene toxicity and the majority of metabolites (Hodson, et al., 2007a). Additionally, partial inhibition of CYP1A alters the abundance and persistence of specific retene metabolites (Hawkins, et al., 2002). This evidence suggests that retene is non-toxic in the absence of metabolism, and it is not the absolute rate of PAH metabolism but the formation of particular metabolites that mediates toxicity.

While PAH metabolism by CYP1A enzymes is well characterized for a few compounds, knowledge of metabolism and the resulting metabolites for other PAH is very limited. The in vitro and in vivo CYP1A metabolism of alkyl-PAH such as retene and 1MP generates a mixture of mono and di-hydroxy metabolites. In trout exposed to alkyl-PAH, most metabolites are mono-hydroxy, in the form of ring (OH-ring) and chain hydroxylated (OH-chain) derivatives, as evident by high performance liquid chromatography (HPLC) analysis of tissue or bile. However, early eluting metabolites are presumed to be conjugated di-hydroxy derivatives based on their UV and mass spectra (Tabash, 2002; Hodson, et al., 2007a; LaVoie, et al., 1981).
To assess the role of metabolism in alkyl-PAH toxicity, 1-methylphenanthrene (1MP), was used as a model compound. This compound is amongst a select few whose metabolites have been previously characterized in fish (Tabash, 2002), and which causes BSD in the early life stages of medaka (Turcotte, et al., 2011). Metabolism of 1MP by CYP1A enzymes generates two main groups of metabolites, OH-ring (phenols), and OH-chain (benzylic alcohols) (Tabash, 2002).

In this study, the chronic toxicity of four OH-ring derivatives and one OH-chain derivative of 1MP to medaka embryos was examined and compared to that of the non-hydroxylated 1MP. The following null hypotheses were tested: (1) there are no differences in toxicity between hydroxylated 1MP derivatives and their non-hydroxylated counterpart; (2) there are no differences in the toxicity between ring and chain-hydroxylated 1MP derivatives; (3) there are no differences in the toxicities of different ring-hydroxylated derivatives and (4) there is no relationship between estimates of K_{ow} and the toxicity of hydroxylated APs. The results of this research identify a link between the toxicity of alkyl-PAH metabolites and the likelihood of their para-hydroxylation via CYP1A metabolism.

2.2 Methods

2.2.1 Experimental Design

The chronic toxicity of five hydroxylated derivatives of 1-methylphenanthrene to embryos of Japanese medaka was measured using a 17-day protocol and compared to that of the non-hydroxylated homologue. To quantify the relative toxicity of the two
metabolite categories, six separate bioassays were conducted (only one chemical tested in each) using medaka embryos. Binary endpoints included the rates of mortality, hatch, and embryos that appeared normal. Non-binary endpoints included severity and BSD indices. Embryos were exposed to the model alkyl-PAH (1MP), each of four OH-ring derivatives: 1-methyl-4-hydroxyphenanthrene (1MP4), 1-methyl-7-hydroxyphenanthrene (1MP7), 1-methyl-8-hydroxyphenanthrene (1MP8), and 1-methyl-9-hydroxyphenanthrene (1MP9), and one (the only possible) OH-chain derivative 1-(hydroxymethyl)phenanthrene (OH-MP) (Figure 2-1).

Figure 2-1 Molecular structures for test compounds. (a) 1-methylphenanthrene (1MP). (b) 1-methyl-4-hydroxyphenanthrene (1MP4). (c) 1-methyl-7-hydroxyphenanthrene (1MP7). (d) 1-methyl-8-hydroxyphenanthrene (1MP8). (e) 1-methyl-9-hydroxyphenanthrene (1MP9). (f) 1-(hydroxymethyl)phenanthrene (OH-MP).
2.2.2 Chemicals and Reagents

Methylphenanthrene was synthesized as described by Cai, et al., (2004), and 1MP4, 1MP7, 1MP8, 1MP9, and OH-MP were synthesized using a modification of the Cai method (Rantanen & Snieckus, Manuscript in preparation). All chemicals except 1MP4 were of >98% purity; 1MP4 was 90% pure, with 1-methyl-9-(propan-2-yl)phenanthren-4-ol comprising the 10% impurity. All compounds were recrystallized after synthesis and purity was confirmed by nuclear magnetic resonance (NMR).

Methanol (HPLC grade; Fisher Scientific, Nepean, ON) was used as a solvent carrier for all chemicals. Ethyl alcohol (anhydrous; Commercial Alcohols Inc, Brampton, ON) was used to preserve water samples. Tricaine methane sulfonate (MS-222; Sigma-Aldrich, Oakville, ON) was used as a fish anaesthetic. The embryo rearing solution (ERS) (1 ml of 10% NaCl, 1 ml of 0.3% KCl, 1 ml of 0.4% CaCl2•2H2O, 1.63% MgSO4•7H2O, and 96 ml of water) was prepared in-house with standard reagents at their best available purity. De-ionized water (18.2 MΩ-cm) was prepared in-house using the PURELAB® Ultra water system (Siemens Water Technologies, Mississauga, ON).

2.2.3 Bioassay Preparation and Characterization

Japanese medaka eggs were collected 0-4 h post fertilization from multiple females in an in-house breeding colony maintained as described by Kirchen and West (1999). Eggs were pooled and placed randomly in 20 ml scintillation vials containing exposure solutions, 2 eggs/15 ml ERS for every scintillation vial, with n=20 eggs for each exposure concentration. Treatments were maintained with a 16L:8D photoperiod at
an average temperature of 26.3±1.6 ºC (Appendix A). Embryo toxicity tests were conducted over a 17 day period and test solutions were renewed daily (24 h static daily renewal).

Test solutions were prepared by the addition of an appropriate volume of a concentrated methanol stock solution to ERS (0.006% v/v). Multiple methanol stock solutions were generated for each test compound, such that exposure solutions of varying chemical concentrations would share the same concentration of the methanol solvent carrier. Additionally, methanol (0.006% v/v) and ERS controls were included to account for any baseline response associated with the solvent carrier and the exposure water respectively.

All treated fish including controls were observed daily for morphological abnormalities under a dissecting microscope. Non-treatment mortality was defined as embryos that terminated before the onset of retinal pigmentation or stage 28 (Iwamatsu, 2004), with no observable signs of toxicity, and those lost or damaged due to transfer. Water samples (3.0 ml) were taken daily and diluted 50:50 with HPLC-grade anhydrous ethanol in a 7 ml glass scintillation vial. Vials were stored at 4°C and analyzed together at the end of each experiment.

Concentrations of each test compound were analyzed by scanning spectrofluorometry (Quanta-Master Fluorescence Spectrometer; PTI Ltd., London, ON). Felix software version 1.4 (Photon Technology International) was used to integrate the fluorescence profile for each sample, which was compared to standard curves prepared
for each chemical. The excitation and emission wavelengths were 299nm and 330-425nm for 1MP, 307nm and 345-425nm for 1MP4, 312nm and 330-450nm for 1MP9, 303nm and 330-420nm for 1MP7, 311nm and 330-420nm for 1MP8, and 298nm and 330-420nm for OH-MP.

2.2.4 Calculating Blue Sac Disease and Severity Indices

Medaka embryos were inspected under a Leica MZ95 (Meyer Instruments, Houston TX) compound microscope, and scored for signs of BSD 17 days after fertilization. Scoring was blind, that is without knowledge of exposure, to minimize bias. Digital images were taken of representative embryos from each treatment using a SPOT RT digital camera and SPOT imaging software (Ver.4.6, Diagnostic Instruments, Sterling Heights, MI, USA). Craniofacial deformities (CF), body axis/spinal deformities (SD), fin rot (FR), ocular edema (OE), and body hemorrhaging (BH) were scored based on presence/absence (0-1). Pericardial edema (PE) and yolk sac edema (YE) were scored based on volume, as a range of edematous fluid accumulation in the respective compartments (0-3). Tube heart (TH) and circulation (CR) were also scored based on severity (0-2). A TH score of 1 indicated impairment in cardiac looping, while a score of 2 indicated a fully stretched heart resembling a string/tube and the absence of ventricular beating. A CR score of 1 indicated a reduction in blood flow velocity, while a score of 2 indicated the absence of visible blood flow.
Toxicity scores were analyzed to generate a severity index (SI), which included mortality and was used to illustrate overall toxicity, and a BSD index, which excluded mortality and was used to highlight the prevalence of individual signs of toxicity.

The SI illustrates the overall observed toxicity on a 0-1 scale. Separate SIs were calculated for pre and post-hatch embryos, as some signs of BSD (CF, SD, FR, and OE) were only observable in hatched embryos. These indices were calculated using a modification of the Villalobos, et al. (2000) equation:

**Pre-hatch:**

\[
SI = \left[ \sum_{i=1}^{n} (PE \cdot E_i) + \sum_{j=1}^{n} (YE \cdot E_j) + \sum_{p=1}^{n} (BH \cdot E_p) + \sum_{q=1}^{n} (TH \cdot E_q) + \sum_{r=1}^{n} (CR \cdot E_r) + (E_D \times 11.5) \right] \\
\div \text{maximum SI score}
\]

**Post-hatch:**

\[
SI = \left[ \sum_{i=1}^{n} (PE \cdot E_i) + \sum_{j=1}^{n} (YE \cdot E_j) + \sum_{p=1}^{n} (BH \cdot E_p) + \sum_{q=1}^{n} (TH \cdot E_q) + \sum_{r=1}^{n} (CR \cdot E_r) + \sum_{k=1}^{n} (CF \cdot E_k) + \sum_{l=1}^{n} (SD \cdot E_l) + \right. \\
\left. + \sum_{m=1}^{n} (FR \cdot E_m) + \sum_{o=1}^{n} (OE \cdot E_o) + (E_D \times 15.5) \right] \div \text{maximum SI score}
\]

where \( E_D \) equals the number of dead embryos, \( E_i \) and \( E_j \) equal the number of embryos displaying a particular severity of PE and YE, \( E_k, E_l, E_m, E_o, \) and \( E_p \) equal the number of embryos displaying a particular severity of CF, SD, FR, OE, and BH, and \( E_q, \) and \( E_r \) equal the number of embryos displaying a particular severity of TH and CR respectively.

In keeping with previous publications (Villalobos, et al., 2000; McIntosh, et al., 2010; and Scott & Hodson, 2008), the score for lethality was set to (0.5) units greater than the most severe case of abnormalities, with values of 11.5 and 15.5 for pre and post-
hatch embryo lethality respectively. The maximum SI score indicated 100% mortality and was the product of the score for lethality and the number of fish in the treatment.

Similar to SIs, BSD scores were summed and divided by the maximum possible score to generate BSD indices. These indices were only calculated for signs that could be observed in both hatched and un-hatched embryos (YE, PE, TH, CR, and BH) and only in treatments with less that 20% mortality, allowing for sufficient data for trends to be observed without the bias inherent in small sample sizes. Using a higher cut off of 50% mortality had no effect on the overall picture of toxicity (Appendix B).

Embryos were also inspected under a dissecting scope for no movement (qualitative mechanosensory test), impaired swim bladder inflation, and 2:1 AV conduction block as described by Incardona, et al., (2004), as well as midbrain (CNS) tissue opacity as described by Incardona, et al., (2008). All embryos passed the mechanosensory test and no 2:1 AV block was observed.

Additionally, while improper hatch (IH), defined as the inability of the embryo to fully emerge from the chorion, impaired swim bladder inflation, and midbrain (CNS) tissue opacity were noted in the highest concentrations of some test treatments (Appendix C), they were not systematically scored, and as a result excluded as a potential toxicity endpoint.

2.2.5 Data Analysis

All endpoints were evaluated using logistic regressions,

\[ Y = Bottom + (Top - Bottom)/(1 + 10^{(\text{Log}_{EC_{50}} - X)\cdot\text{Hill Slope}}), \]

with constraints of 0
and 100 for percentage response, and 0 and 1 for indices. Non-linear regressions (sigmoidal concentration-response curves), median lethal concentrations (LC50s), median effective concentrations (EC50s), median effective time (ET50s), and t-tests were analyzed and calculated using GraphPad Prism software (Ver. 5, GraphPad Software, Inc., San Diego, CA, USA).

There was no statistical difference between the two control treatments (water and solvent) as determined by a two-tailed t-test (unpaired) for any of the analyzed endpoints, % hatch (P=0.19), BSD (P=0.06), % normal (P=0.45), and ET50Hatch (P=0.20). As a result ERS (n=6) and MeOH (n=6) controls from all bioassays were pooled (n=12) to create one negative control category. Median effect/lethal values were only used for chemicals that induced at least a 50% effect, as they otherwise carried great uncertainty. Chemicals were compared on a molar basis due to the difference in molar mass between 1MP and its derivatives.

2.3 Results

2.3.1 Chemical Exposures

Concentrations of test chemicals varied over the 24h static daily renewal. Waterborne concentrations after 24 hours (T24), differed from concentrations measured immediately following each chemical’s addition (T0), with an average of 12-19% decrease (n=144 for each chemical). The OH-chain derivative was an outlier, with an average of 37% change in waterborne concentrations over 24 h. These 24 h changes are consistent with similar experiments which reported a range of 20% (Scott et al, 2009) to
59% (Scott and Hodson, 2008) change. However, while there was variance in measured concentrations, there was no significant trend over the course of the entire experiment (P>0.05). As a result, T0 and T24 measurements were averaged for each exposure concentration over the 17d period to generate one representative value for the measured exposure concentration.

2.3.2 Chronic Toxicity – Embryo Mortality

An exposure-related increase in percent mortality was observed in embryos exposed to all OH-ring derivatives (Figure 2-2a), with similar median lethal concentrations (LC50s) and overlapping 95% confidence intervals (CIs) (Table 2-1). Mortality in embryos exposed to the reference compound (1MP) and the OH-chain derivative (OH-MP) exceeded control mortality, but did not surpass 12%. These compounds were less toxic than OH-ring derivatives, and mortality was insufficient for calculating LC50s.
Table 2-1 The rank order of toxicity (Rank), median lethal (LC50) and effective concentrations (EC50s) for Japanese medaka embryos exposed for 17d to six alkyl-phenanthrenes. Concentrations are presented in molar units, followed by mass per volume. 1MP is 1-methylphenanthrene; 1MP4 is 1-methyl-4-hydroxyphenanthrene; 1MP7 is 1-methyl-7-hydroxyphenanthrene; 1MP8 is 1-methyl-8-hydroxyphenanthrene; 1MP9 is 1-methyl-9-hydroxyphenanthrene; OH-MP is 1-(hydroxymethyl)phenanthrene.

<table>
<thead>
<tr>
<th>PAH, (Rank)</th>
<th>LC50 (95%CI) (µmol/L), (µg/L)</th>
<th>EC50s (95%CI) (µmol/L), (µg/L)</th>
<th>EC50Normal (95%CI) (µmol/L), (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1MP, (2)</td>
<td>&gt; 1.42, &gt; 273</td>
<td>1.42 (1.05-1.92), 272 (202-368)</td>
<td>0.973 (0.857-1.10), 187(165-212)</td>
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<td>1MP4, (3)</td>
<td>0.447 (0.406-0.492), 93 (85-102)</td>
<td>0.425 (0.362-0.447), 84 (76-93)</td>
<td>1.03 (0.922-1.14), 214 (192-238)</td>
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<tr>
<td>1MP7, (1)</td>
<td>0.367 (0.339-0.397), 76 (71-83)</td>
<td>0.286 (0.227-0.360), 60 (47-75)</td>
<td>&gt; 0.566, &gt; 118</td>
</tr>
<tr>
<td>1MP8, (1)</td>
<td>0.393 (0.184-0.838), 82 (38-174)</td>
<td>0.356 (0.272-0.465), 74 (57-97)</td>
<td>&gt; 0.988, &gt; 206</td>
</tr>
<tr>
<td>1MP9, (3)</td>
<td>0.390 (0.177-0.859), 81 (37-179)</td>
<td>0.386 (0.300-0.498), 80.5 (62-104)</td>
<td>0.861 (0.649-1.14), 179 (135-238)</td>
</tr>
<tr>
<td>OH-MP, (3)</td>
<td>&gt; 0.648, &gt; 135</td>
<td>&gt; 0.648, &gt; 135</td>
<td>&gt; 0.648, &gt; 135</td>
</tr>
</tbody>
</table>

0.081 (0.050-0.132), 17 (10-28)
Figure 2-2 The response of medaka embryos (n=20) to a 17-day exposure to six alkyl-phenanthrenes, expressed as (a) % mortality, (b) severity index (SI), (c) % hatch, and (d) % normal. There was no control mortality and grey bands represent the 95% confidence intervals for responses of negative controls. 1MP is 1-methylphenanthrene and presented as a solid line. Hydroxylated derivatives are presented as dashed lines. 1MP4 is 1-methyl-4-hydroxyphenanthrene; 1MP7 is 1-methyl-7-hydroxyphenanthrene; 1MP8 is 1-methyl-8-hydroxyphenanthrene; 1MP9 is 1-methyl-9-hydroxyphenanthrene; OH-MP is 1-(hydroxymethyl)phenanthrene.
2.3.3 Chronic Toxicity – Severity and BSD Indices

Two indices were used to evaluate toxicity. The severity index included both sub-lethal effects and mortality, and was used to provide an overall picture of toxicity, while the BSD index included only sub-lethal signs of toxicity and was used to highlight differences among chemicals. An exposure related increase in overall toxicity as indicated by SI (i.e., mortality + BSD) was observed in embryos exposed to all test compounds (Figure 2-2b). As with mortality, similar toxicity was observed among all OH-ring derivatives, based on median effective concentrations (EC50SI) and overlapping 95% CIs (Table 2-1). These derivatives were more toxic than the OH-chain derivative which did not reach a 50% effect, and 3-5 times more toxic than the reference compound.

Using the BSD index test compounds could be classified into three groups based on the prevalence of individual signs of toxicity (Figure 2-3). Group 1: (1MP) based on the presence of CR, YE, and a small PE response, and the similar prevalence of CR and YE. Group 2: (1MP7, 1MP8) based on the presence of CR, YE, and PE, coupled with the relative minor prevalence of PE as compared to CR and YE. Group 3: (1MP4, 1MP9, OH-MP) based on the overall dominance of CR.
Figure 2-3 The prevalence of individual signs of blue sac disease (BSD); yolk sac edema (YE), pericardial edema (PE), tube heart (TH), circulation (CR), and body hemorrhaging (BH) observed in both hatched and un-hatched embryos for each test compound. 1MP is 1-methylphenanthrene; 1MP4 is 1-methyl-4-hydroxyphenanthrene; 1MP7 is 1-methyl-7-hydroxyphenanthrene; 1MP8 is 1-methyl-8-hydroxyphenanthrene; 1MP9 is 1-methyl-9-hydroxyphenanthrene; OH-MP is 1-(hydroxymethyl)phenanthrene.
2.3.4 Chronic Toxicity – Embryo Hatching Success

A similar decrease in hatching success was observed for 1MP, 1MP4, and 1MP9 (Figure 2-2c), which shared similar EC50s and 95% CIs (Table 2-1). Hatching success was also affected in embryos exposed to 1MP7 and 1MP8 and exceeded that of control treatments, but the effects were insufficient for the calculation of EC50s. Hatching success was not affected by OH-MP exposure at tested concentrations. While time to hatch varied between 8 and 17 days post fertilization (dpf), the median time to hatch (ET50) was only affected in embryos exposed to the highest concentrations of 1MP and 1MP4 (Figure 2-4).

![Figure 2-4](image.png)

**Figure 2-4** The median effective time to hatch (ET50\textsubscript{Hatch}) for medaka embryos (n=8/ET50) exposed to six alkyl-phenanthrenes for 17 d. The grey bands represent 95% confidence intervals for responses of negative controls. 1MP is 1-methylphenanthrene and presented as a solid line. Hydroxylated derivatives are presented as dashed lines. 1MP4 is 1-methyl-4-hydroxyphenanthrene; 1MP7 is 1-methyl-7-hydroxyphenanthrene; 1MP8 is 1-methyl-8-hydroxyphenanthrene; 1MP9 is 1-methyl-9-hydroxyphenanthrene; OH-MP is 1-(hydroxymethyl)phenanthrene.
2.3.5 Chronic Toxicity - % Normal

Embryos without signs of toxicity were classed as “normal”. An exposure related decrease in the percentage of normal embryos was observed for all PAH tested (Figure 2-2d). Based on % normal, test chemicals were grouped into three distinct groups according to similar EC50s: group1: 1MP, group 2: 1MP7, 1MP8, and group3: 1MP4, 1MP9, and OH-MP. The most toxic group (group 2) included two OH-ring derivatives, and was 2-3 times more toxic than group 1, which only contained the reference non-hydroxylated compound, and 5-8 times more toxic than group 3 which included two OH-ring and the only OH-chain derivative. This grouping was the same as the proposed grouping of test compounds in accordance to the presence and the prevalence of observed signs of BSD.

2.3.6 Structure and Toxicity:

There was no relationship between the properties of test compounds (Table 2-2) such as K_{ow}, solubility, and the acid dissociation constant (pKₐ), and toxicity. Despite the 5-8 fold difference in toxicity, these descriptors were not useful predictors of toxicity (% mortality, SI, % hatch, and % normal) (Appendix D).
Table 2-2 Properties of test compounds; molecular weights (MW), octanol-water partition coefficients (K_{ow}), and solubility. Estimates were generated using the Sparc Performs Automated Reasoning in Chemistry (SPARC) software (Ver. 4.5).

<table>
<thead>
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<th>Short Form</th>
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<th>Estimates</th>
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<td>1-methylphenanthrene</td>
<td>1MP</td>
<td>192.26</td>
<td>5.16</td>
</tr>
<tr>
<td>1-methyl-4-hydroxyphenanthrene</td>
<td>1MP4</td>
<td>208.26</td>
<td>4.97</td>
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<td>1-methyl-7-hydroxyphenanthrene</td>
<td>1MP7</td>
<td>208.26</td>
<td>5.07</td>
</tr>
<tr>
<td>1-methyl-8-hydroxyphenanthrene</td>
<td>1MP8</td>
<td>208.26</td>
<td>4.99</td>
</tr>
<tr>
<td>1-methyl-9-hydroxyphenanthrene</td>
<td>1MP9</td>
<td>208.26</td>
<td>4.99</td>
</tr>
<tr>
<td>1-(hydroxymethyl)phenanthrene</td>
<td>OH-MP</td>
<td>208.26</td>
<td>3.91</td>
</tr>
</tbody>
</table>

2.4 Discussion

The results of this study indicate that hydroxylated derivatives of 1MP differ in toxicity from their non-hydroxylated counterpart. Ring hydroxylation can increase PAH toxicity, as some 1MP phenol derivatives were more toxic than 1MP itself and its benzylic alcohol derivative. These data suggest that metabolism may enhance alkyl-PAH toxicity through the generation of OH-ring hydroxylated metabolites. Additionally, OH-ring derivatives substituted in the 1,7 and 1,8 positions (1MP7 and 1MP8) were the most toxic tested, and closely mimicked the prevalence of the signs of toxicity observed in the non-hydroxylated reference compound. The rank order of toxicity for exposure chemicals was endpoint dependent, highlighting the importance of multiple endpoints in determining relative toxicity. The 5 to 50-fold difference between LC50 and EC50 values reflects the crudeness of mortality as a toxicity endpoint. These data also suggest that the effects and likely mechanisms of toxicity are not uniform among closely related structures.
Analysis of mortality and SI indices point to a categorical difference in the toxicity of phenol derivatives as compared to the non-hydroxylated and chain hydroxylated 1MP. The increased toxicity of this group may relate to more general differences in solubility and the degree of substitution among the tested compounds. The OH-ring compounds were predicted to be more soluble than the non-hydroxylated 1MP, and less soluble than the OH-chain derivative. Additionally, this group also contained only di-substituted PAHs.

The data on hatching success suggest that this parameter was not a sensitive indicator of toxicity for test chemicals. While some metabolite candidates caused an exposure-related adverse effect on hatching success similar to that of the parent compound, hatching success and time to hatch were unaffected at concentrations causing other important embryonic effects, including lethality both pre and post-hatch.

Preceded by the opercular movement of the embryo, the active hatching enzyme (chorionase) is secreted by the hatching glands, and digests the majority of the inner layer of the chorion (egg envelope). The quantity of enzyme secreted by the embryo is more than sufficient for the digestion of a single chorion (Yamagami, 1981), and has been proposed adequate for the dissolution of up to 15 eggs (Moriwaki, 1910). Once the embryo has hatched, the enzyme is released into the surrounding water. For static daily renewal protocols where the volume of exposure water is typically no more than 7ml/egg, the excess enzyme released into the exposure water as a result of hatch could act as a confounding factor by initiating hatch on the same day in surrounding embryos. This
factor was not a major consideration in this experiment due to the isolation of embryos (n=2 per scintillation vial).

Concurrent examination of % normal and the prevalence of signs of BSD strongly suggest three groupings amongst exposure chemicals and may indicate differences in mechanisms of action. Embryonic exposure to the non-hydroxylated reference compound (group 1) lead primarily to circulatory effects and the prevalence of edema. Similarly, exposure to the 1,7 and 1,8 substituted phenol derivatives (group 2) lead to the same embryonic abnormalities, which may indicate that group 1 and 2 compounds are toxic via the same mode of action. However, group 3 compounds mainly caused circulatory effects, with only a minor prevalence of edema. Edema has been suggested as the most important indicator of PAH toxicity (Carls, et al., 1999), and its absence in embryos exposed to group 3 compounds may account for the higher percentage of normal larvae present in these treatments.

The poor performance of K_{ow}, solubility, and pKa as predictors of toxicity could relate to the inability of quantitative structure activity relationship (QSAR) based software to distinguish between OH-PAH covering a very narrow range of these descriptors, with the same molecular components and subtle structural differences (Appendix D). Typically, these estimates are based primarily on the presence of particular fragments, with subtle structural differences making a relatively small contribution to the overall value (Meylan & Howard, 2000). In dealing with structurally similar compounds, fragment based methods can run the risk of the oversimplification of
steric and conformational effects (Howard & Meylan, 1997). In particular, the 5 to 8-fold difference in toxicity in test chemicals is not explained by \(K_{ow}\) estimates. This is inconsistent with narcosis as the mode of toxic action (Di Toro, et al., 2000), and instead suggests the potential involvement of specific biological action in the observed toxicity.

2.4.1 Proposed Mechanism of Toxicity

The results of this study are consistent with a link between OH-PAH toxicity and the preferential formation of \(para\)-quinones by the most toxic grouping. In examining the five tested OH-PAH, the differences in toxicity may relate to the likelihood of \(para\)-hydroxylation via further CYP1A metabolism. The most toxic metabolite candidates tested, 1MP7 and 1MP8, can be \(para\)-hydroxylated to generate diols and subsequently diones (Figure 2-5a,b). However in the less toxic OH-ring derivatives, 1MP4 and 1MP9, the \(para\)-hydroxy position is unavailable. In the case of 1MP4, the \(para\) position is blocked by the methyl group, and no true \(para\)-substitution position exists for 1MP9. The least toxic chemical tested, OH-MP, differs more generally from the OH-ring compounds, as it is most likely to become an aldehyde, then further oxidized to generate a carboxylic acid, and subsequently excreted (Figure 2-5c).
This study also makes an interesting linkage to earlier work relating the inhibition of the 9,10 dihydro-diol metabolite of 1MP and mutagenicity (LaVoie, et al., 1981). The 9,10 diol is not mutagenic, cannot become \textit{para}-hydroxylated, and will not form \textit{para}-quinones, which may have a broader implication for the role of \textit{para}-quinones in toxicity.

2.4.2 Summary

The mechanisms of PAH and alkyl-PAH toxicity are not well understood. Existing literature suggests that the addition of hydroxyl groups to PAH and the subsequent decrease in log $K_{ow}$, should result in decreased toxicity. The results of this study propose that the variability observed with respect to alkyl-PAH toxicity is not simply a function of hydrophobicity, but potentially a more specific response to the preferential formation of particular metabolites. These findings highlight 1,7 and 1,8

\begin{figure}[h]
\centering
\includegraphics{figure2-5.png}
\caption{The proposed \textit{para}-hydroxylation pathway for \textbf{a)} 1-methyl-7-hydroxyphenanthrene (1MP7) and \textbf{b)} 1-methyl-8-hydroxyphenanthrene (1MP8); \textbf{c)} the proposed excretory pathway for 1-(hydroxymethyl)phenanthrene (OH-MP).}
\end{figure}
substituted alkyl-phenanthrene metabolites as having both higher toxicity, and a higher preference for para-quinone formation.

2.5 Acknowledgements

This research was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) to Peter V. Hodson. The authors would like to thank Helen Waters, Angie Le Beau, and all the past and present members of the Hodson and Brown Labs for their contributions to this project.
2.6 References


Chapter 3

General Discussion and Summary

3.1 Overview

Alkyl-Phenanthrenes (APs) are common aquatic contaminants found at industrially contaminated sites (Leppanen & Oikari, 1999), and have been identified as the principal component of oil that is chronically toxic to the early life stages of fish (Khan, 2007). Alkyl-PAH concentrations can be used to assess damage to natural resources following oil spills (Incardona, et al., 2008) or effluent release (Leppanen & Oikari, 1999), and are often the focus of remedial activities.

The mechanisms of PAH and alkyl-PAH toxicity are not well understood. While most PAH are predicted to act via narcosis, the results presented here, particularly the lack of correlation between variations in the estimated octanol-water partition coefficients ($K_{ow}$) and differences observed in the toxicity of test chemicals (Table 2-1) suggests that narcosis is not responsible for the observed toxicity (Di Toro, et al., 2000). Additionally, all affected embryos passed the qualitative mechanosensory test (no movement) and exhibited an exposure-related increase in signs of blue sac disease, a non-contagious disease associated with receptor mediated cytochrome P4501A (CYP1A) induction (Bauder, et al., 2005). This information highlights the importance of considering the potential specific biological activity of PAH in risk assessment in schemes.
The enzymatic metabolism of APs generates ring (OH-ring) and chain hydroxylated (OH-chain) derivatives (Tabash, 2002), and has been associated with the increased prevalence of toxicity in early life stages (ELS) of fish (Hawkins, et al., 2002). The role of PAH metabolism in toxicity remains unclear, and may involve the byproducts of metabolism such as reactive oxygen species (ROS), reactive intermediates, metabolites themselves, or a combination thereof. While the prevalence of toxicity may relate to the formation of particular metabolite classes, information regarding the identity of PAH metabolites is scarce, and data on their toxicity is non-existent.

Previous work suggests that AP toxicity is not caused solely by the byproducts of metabolism such as reactive oxygen species (ROS), as the prevalence of toxicity is incongruent with the rate of AP metabolism and therefore ROS formation (Tabash, 2002). Alternatively, if AP toxicity is metabolite mediated, the prevalence of toxicity may be linked to the formation of particular metabolite classes.

The working hypothesis of this thesis has been that AP toxicity is mediated by CYP1A metabolism and the formation of hydroxylated metabolites. The simplest AP, 1-methylphenantherene (1MP), was used as a model compound to answer the following null hypotheses: (1) no difference in toxicity between hydroxylated 1MP derivatives and their non-hydroxylated counterpart; (2) no difference in toxicity between ring and chain-hydroxylated 1MP derivatives; (3) no differences in toxicity among different ring-hydroxylated derivatives and (4) no relationship between Kow estimates and the toxicity of hydroxylated APs.
The results of this study indicate that ring-hydroxylation can lead to increased alkyl-PAH toxicity, while chain-hydroxylation can decrease toxicity. Similarities between some CYP1A metabolite candidates and the parent compound in the nature and prevalence of toxic signs, provides a strong basis for their involvement in alkyl-PAH toxicity. Additionally, the higher toxicity of some hydroxylated test compounds highlights the potential limitations of narcosis-based modeling in assessing the risk of PAH derivatives to fish.

3.2 Significance of Findings and Future Work

Understanding the toxicity of AP metabolites was the primary research objective of this thesis. Previous information on the identity of alkyl-PAH metabolites generated using High Pressure Liquid Chromatography (HPLC), fluorescence photodiode array (PDA), and mass spectroscopy (MS) was categorical, classifying metabolites into ring hydroxylated, chain hydroxylated and further oxidized (Tabash, 2002). Given that the location of ring-hydroxylation can affect toxicity, identifying preferential hydroxylation sites using modeling software can assist in the interpretation of the toxicity data.

It is important to note that the results presented in this thesis cannot clarify whether the higher toxicity associated with the tested ring-hydroxylated PAH is due to the byproducts of metabolism such as ROS, or a direct receptor mediated effect. Examination of embryos exposed to ring-hydroxylated PAH derivatives using \textit{in vivo} ethoxyresorufin O-deethylase (EROD) analysis may provide insight as to whether these compounds are further metabolized by the CYP1A pathway.
This research provides new information pertaining to the risk of PAH in aquatic ecosystems, illustrating a difference in toxicity among 1MP, and its derivatives. This information suggests that particular PAH sub-classes, such as phenol derivatives will be more toxic to fish, and emphasizes the potential for enzymatic metabolism to enhance PAH toxicity. These data highlight the need for the development of analysis standards for oxygenated PAH, to allow for a more thorough assessment of risk at contaminated sites.
3.3 Summary of Findings

1. The toxicity of hydroxylated 1MP derivatives is endpoint dependent.

2. Hydroxylated derivatives of 1MP differ in toxicity from 1MP.

3. Ring hydroxylated derivatives of 1MP differ in toxicity from chain hydroxylated derivatives.

4. Ring hydroxylation can increase PAH toxicity, and different ring hydroxylated derivatives of 1MP can differ in toxicity.

5. Ring hydroxylated 1MP derivatives that are likely to form \textit{para}-quinones were also most toxic to the early life stages of medaka.

6. Hatching success was not a sensitive indicator of toxicity for test chemicals.

7. Descriptors such as $K_{ow}$, solubility, and pKa did not account for differences in toxicity among test chemicals.

8. The toxicity of hydroxylated 1MP derivatives was not consistent with a narcotic mode of action.

9. The effects, and likely mechanisms of PAH toxicity are not uniform among closely related structures.

10. Risk assessment schemes would benefit from considering the potential role of specific biological activity in assessing alkyl-PAH toxicity.
3.4 References


Appendix A

Daily Temperature Logs

Table 1 Daily water temperature (T) logs for each test compounds. 1MP is 1-methylphenanthrene; 1MP4 is 1-methyl-4-hydroxyphenanthrene; 1MP7 is 1-methyl-7-hydroxyphenanthrene; 1MP8 is 1-methyl-8-hydroxyphenanthrene; 1MP9 is 1-methyl-9-hydroxyphenanthrene; OH-MP is 1-(hydroxymethyl)phenanthrene.

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Appendix B

Evaluation of BSD Indices Using a Cut off of 50% Mortality

Figure 1 The prevalence of yolk sac edema (YE), pericardial edema (PE), tube heart (TH), circulation (CR), and body hemorrhaging (BH) observed in both hatched and un-hatched embryos for each test compound re-evaluated using a 50% cut off for mortality. 1MP is 1-methylphenanthrene; 1MP4 is 1-methyl-4-hydroxyphenanthrene; 1MP7 is 1-methyl-7-hydroxyphenanthrene; 1MP8 is 1-methyl-8-hydroxyphenanthrene; 1MP9 is 1-methyl-9-hydroxyphenanthrene; OH-MP is 1-(hydroxymethyl)phenanthrene. The re-evaluation of results using a less stringent cut off did not change any of the overall conclusions regarding toxicity.
Appendix C

Excluded Toxicity Endpoints

<table>
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**Figure 1** Excluded toxicity endpoints: improper hatch, impaired swim bladder (SB) inflation, and midbrain tissue opacity. (A) Normal embryo, successful hatch; (B) embryo exposed to 125µg/l of 1-methyl-9-hydroxyphenanthrene (1MP9); improper hatch (IH), defined as the inability of embryos to fully emerge from the chorion; (C) normal embryo with inflated swim bladder; (D) embryo exposed to 135µg/l of 1-(hydroxymethyl)phenanthrene (OH-MP); failure to inflate swim bladder; (E) normal embryo without midbrain tissue opacity; (F) embryo exposed to 118µg/l of 1-methyl-7-hydroxyphenanthrene (1MP7); presence of midbrain tissue opacity.

Scale bar = 1.0mm.
Appendix D

**K\textsubscript{ow}, Solubility, and pKa as Descriptors of Toxicity**

**Table 1** Estimates of octanol-water partition coefficients K\textsubscript{ow} values using predictive software: ALOGPS (Ver. 2.1), EPI (Ver. 4.0), SPARC (Ver. 4.5), and ADMET predictor (Ver. 5.0). 1MP is 1-methylphenanthrene and presented as a solid line. Hydroxylated derivatives are presented as dashed lines. 1MP4 is 1-methyl-4-hydroxyphenanthrene; 1MP7 is 1-methyl-7-hydroxyphenanthrene; 1MP8 is 1-methyl-8-hydroxyphenanthrene; 1MP9 is 1-methyl-9-hydroxyphenanthrene; OH-MP is 1-(hydroxymethyl)phenanthrene.

<table>
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<tr>
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<th>SPARC</th>
<th>ADMET</th>
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<td>3.53</td>
<td>3.43</td>
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**Table 2** Estimates of water solubility using predictive software: ALOGPS (Ver. 2.1), EPI (Ver. 4.0), SPARC (Ver. 4.5), and ADMET predictor (Ver. 5.0). 1MP is 1-methylphenanthrene and presented as a solid line. Hydroxylated derivatives are presented as dashed lines. 1MP4 is 1-methyl-4-hydroxyphenanthrene; 1MP7 is 1-methyl-7-hydroxyphenanthrene; 1MP8 is 1-methyl-8-hydroxyphenanthrene; 1MP9 is 1-methyl-9-hydroxyphenanthrene; OH-MP is 1-(hydroxymethyl)phenanthrene.

<table>
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<td>OH-MP</td>
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Table 3 Estimates of acid dissociation constant (pKa) values using predictive software: ALOGPS (Ver. 2.1), EPI (Ver. 4.0), SPARC (Ver. 4.5), and ADMET predictor (Ver. 5.0). 1MP is 1-methylphenanthrene and presented as a solid line. Hydroxylated derivatives are presented as dashed lines. 1MP4 is 1-methyl-4-hydroxyphenanthrene; 1MP7 is 1-methyl-7-hydroxyphenanthrene; 1MP8 is 1-methyl-8-hydroxyphenanthrene; 1MP9 is 1-methyl-9-hydroxyphenanthrene; OH-MP is 1-(hydroxymethyl)phenanthrene.

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Figure 1 The relationship between 17day median lethal (LC50) and effective (EC50) concentrations (n=8) for medaka embryos, and the octanol-water partition coefficients (K_{ow}) of six alkyl-phenanthrenes. No significant linear trends could be calculated, and K_{ow} estimates were generated using the SPARC predictive software (Ver. 4.5). 1MP is 1-methylphenanthrene; 1MP4 is 1-methyl-4-hydroxyphenanthrene; 1MP7 is 1-methyl-7-hydroxyphenanthrene; 1MP8 is 1-methyl-8-hydroxyphenanthrene; 1MP9 is 1-methyl-9-hydroxyphenanthrene; OH-MP is 1-(hydroxymethyl)phenanthrene.
Figure 2 The relationship between 17-day median lethal (LC50) and effective (EC50) concentrations (n=8) for medaka embryos, and solubility (S) of six alkyl-phenanthrenes. No significant linear trends could be calculated. Solubility estimates were generated using the SPARC predictive software (Ver. 4.5). 1MP is 1-methylphenanthrene; 1MP4 is 1-methyl-4-hydroxyphenanthrene; 1MP7 is 1-methyl-7-hydroxyphenanthrene; 1MP8 is 1-methyl-8-hydroxyphenanthrene; 1MP9 is 1-methyl-9-hydroxyphenanthrene; OH-MP is 1-(hydroxymethyl)phenanthrene.
Figure 3 The relationship between 17day median lethal (LC50) and effective (EC50) concentrations (n=8) for medaka embryos, and the acid dissociation constant (pKa) of six alkyl-phenanthrenes. No significant linear trends could be calculated, and pKa estimates were generated using the SPARC predictive software (Ver. 4.5). 1MP is 1-methylphenanthrene; 1MP4 is 1-methyl-4-hydroxyphenanthrene; 1MP7 is 1-methyl-7-hydroxyphenanthrene; 1MP8 is 1-methyl-8-hydroxyphenanthrene; 1MP9 is 1-methyl-9-hydroxyphenanthrene; OH-MP is 1-(hydroxymethyl)phenanthrene.