
By

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Abstract
Polycyclic aromatic hydrocarbons (PAH) are hydrophobic environmental contaminants known to impact survival and reproduction in fish species. This study tested the toxicity of benz[a]anthracene (B[a]A) and 7-methylbenz[a]anthracene (7-MeB[a]A) to early life stages of Japanese medaka (Oryzias latipes) using the partition controlled delivery (PCD) method of exposure and the relationship between toxicity and the log of octanol-water partition coefficient (log P). The exposure method PCD provides stable exposure concentrations maintained at and below the solubility limit of test chemicals throughout chronic toxicity assays. It relies on the partitioning of the test chemical from prepared poly (dimethylsiloxane) (PDMS) films, loaded with various concentrations of chemical, to the exposure solution. Aqueous solubility limits and film: solution partition coefficients (log K_{fs}) were determined. The prevalence of blue sac disease (BSD) in exposed medaka embryos was used to determine median effective concentration (EC50) of the two congeners. The test chemical 7-MeB[a]A was more toxic than B[a]A, and toxicity increased with log P. These results will further contribute to models for assessing the risk of PAH mixtures as well as to our understanding of how toxicity is affected by alkylation.
Acknowledgements

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**Introduction**

Crude oil is a complex mixture of hydrocarbons, including straight chain alkanes and polycyclic aromatic hydrocarbons (PAH), resins and asphaltenes. PAH are compounds composed of a minimum of two fused aromatic rings and have been shown to contribute to crude oil toxicity (Heintz et al., 1999). Following the Exxon Valdez oil spill, 1989, crude oil and its weathered products persisted in the environment for over a decade, contaminating ecologically sensitive habitats and exposing local marine populations to petroleum hydrocarbons (Peterson et al., 2003). Among species affected, early life stages (ELS) of pink salmon (*Oncorhynchus gorbuscha*) and Pacific herring (*Clupea pallasi*) presented signs of Blue Sac Disease (BSD), impairing embryo survival and recruitment. As a result of these long-term ecological effects, research into the components of oil and their relative toxicity has become an area of interest (Incardona et al., 2006; Barron et al., 2004; Billiard et al., 2008).

The BSD syndrome is a non-contagious, chemically-induced disease characterized by a variety of symptoms including: yolk sac and pericardial edema, hemorrhaging, craniofacial deformities and spinal curvature, as well as increased activity of cytochrome P450 1A (CYP1a) enzymes following binding of PAH to the aryl hydrocarbon receptor (AhR) (Billiard et al., 1999). Some PAH and many planar halogenated aromatic compounds such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), are chemicals whose toxicity is mediated through the AhR pathway, and they are known to induce BSD like symptoms (Billiard et al., 1999). When CYP1a potencies were compared among various PAH, un-substituted 2-3 ringed PAH were relatively inactive while 4-6 ringed PAH and alkyl PAH, were associated with higher potency, suggesting a greater ability to induce CYP1a metabolizing enzymes (Barron et al., 2004).
In studies using effects-driven fractionation of Alaska North Slope Crude (ANSC), classes of compounds responsible for CYP1a induction and toxicity were identified (Hodson et al., 2007). These experiments found that PAH were present in all highly toxic fractions derived from the oil; particularly toxic were fractions containing alkylated 3-4 ringed PAH. Conversely, fractions causing the most CYP1a induction contained larger proportions of un-substituted 5-6 ringed PAH but were less toxic. PAH have varying CYP1a potencies and chronic toxicity in exposed fish species.

The mechanistic pathway of PAH metabolism and toxicity has not been fully characterized, but is generally recognized as AhR mediated (Bols et al., 1999). Metabolism is initiated when PAH bind to the cytosolic Ah-receptors, the receptor- ligand complex translocates into the nucleus and binds to the aryl hydrocarbon nuclear translocator (ARNT) protein, enabling activation of the dioxin- response element in the CYP1a gene. Gene activation causes transcription and translation of the CYP1a protein in the cytosol. The CYP1a protein family catalyzes phase 1 oxygenation by adding a oxygen to double bonds. Further reduction to a hydroxyl group produces reactive metabolites including reactive oxygen species (ROS) (Tuvikene, 1995). PAH toxicity has been shown to be metabolite-mediated (Aas et al., 2000, Cavalieri & Rogan, 1985). Following phase-1 activation, ROS and reactive metabolites can be subject to redox cycling facilitating their interaction with macromolecules. These metabolites can induce molecular damage including lipid peroxidation, oxidative modification of amino acids, direct DNA modification and altered protein expression/ activity (Billiard et al., 2002). Among current toxicity models, AhR agonism correlates well with ELS responses but under- predicts toxicity substantially (Barron et al., 2004). The narcosis model generates toxicity values predicted from hydrophobicity but does not predict PAH toxicity accurately (Di Toro & McGrath, 2000). Models based on alkyl-
phenanthrene toxicity trends were able to predict lethal and sub lethal effects in herring and pink salmon from complex mixtures.

Alkylated PAH comprise an estimated 90% of the total PAH in oil and can be more chronically toxic to fish embryos than their un-substituted compounds (Radke et al., 1990). Retene (7-isopropyl-1-methylphenanthrene) is many times more toxic than phenanthrene (Phen) (Turcotte et al., 2011). In the previous effects driven oil fractionation study, alkyl 3-4 ringed PAH such as alkyl fluorenes, Phen, and naphthobenzothiophenes, were implicated as the most likely contributors to toxicity of the whole oil (Hodson et al., 2007). The relationship between toxicity and degree of alkylation is not well characterized. If the presence of these alkylated PAH can enhance oil toxicity, an understanding of their contribution is essential to quantifying PAH mixture effects.

When released into the environment, oil is subject to degradation through evaporation, dispersion, dissolution, and sedimentation. Throughout these processes, low molecular weight (LMW) components rapidly volatize, shifting the composition of oil towards higher proportions of medium and higher molecular weight (MMW, HMW) compounds (Fieldhouse et al., 2003). This change is reflected in the composition of PAH mixtures as LMW 2-ringed PAH volatilize rapidly leaving the MMW 3-5 ringed PAH; this change has been shown to increase oil toxicity (Heintz et al., 1999). These MMW compounds persist after a spill, and are small enough to be readily accumulated by aquatic species, whereas more persistent HMW PAH of 5 or more rings are quite prevalent but have limited toxicity due to low solubility and exposure kinetics.

Due to the low solubility and hydrophobic nature of PAH, traditional embryo toxicity assays have proven difficult to adapt. Static and semi-static exposure protocols have traditionally been
conducted using nominal toxicant concentrations and can lead to inaccurate estimation of toxicity as significant differences can exist between measured and nominal exposure concentrations for many hydrophobic compounds. Egg injection or topical application of chemicals ensures direct exposure but are based on one dose of test chemical and assume chemical can effectively partition through the chorion, implying a relationship between log P and toxicity (Brown et al., 2003). Flow-through systems require excessive amounts of chemical, are inherently complex, and generate large volumes of waste (Brown et al., 2003). The PCD exposure method has been developed to test hydrophobic compounds such as PAH to fish. This method provides reliable and stable concentrations over multi-day exposures at and below the solubility limit of a chemical. It effectively compensates for chemical loss, requires small amounts of test chemical, and can be modified to perform ELS chronic toxicity assays (Brown et al., 2003).

Fish ELS toxicity assays are short in duration (approximately 2 weeks) and provide a conservative estimate of toxicity due to heightened sensitivity compared to other life-stages (Parrott & Teather (2006), Grey et al., (1999)). Japanese medaka have been used in the past due to rapid generation time for females and their ability to produce 10-30 eggs daily. The embryos are transparent and thus facilitate easy evaluation of fertilization success and development (Leaf et al., 2011). The PCD method has been applied to test the chronic toxicity of alkyl-phenanthrenes, and it delivered exposure concentrations at and below predicted water solubility limits and generated EC50 values below solubility (Turcotte et al., 2011). The use of PCD for hydrophobic chemicals is an inexpensive method for the reliable generation of ecologically significant exposure concentrations for chronic toxicity bioassays.
Fluorescence spectroscopy measures the emission of light against a dark background. This analytical method is many times more sensitive for detecting PAH than absorbance (Harris, 2004). Fluorescence detectors can be optimized for specific chemicals through the selection of appropriate emission and excitation wavelengths (Dong et al., 2005) and provides an effective analytical tool for determining individual PAH concentrations when combined with analytical standards.

Current methods for assessing the risk of oil toxicity are unreliable and cannot accurately assess complex mixtures of PAH. In the past, parameters such as the octanol-water partition coefficient (log Kow), toxic equivalency factors (TEF) and total PAH (tPAH) have been employed but do not accurately characterize toxicity (Billiard et al., 2008). Log Kow models based on acute toxicity assays may underestimate toxicity of organic compounds. If log Kow exceeds 3.5, toxicity may be kinetically limited, requiring extended periods of time for these chemicals to accumulate and toxicity to become apparent. Implementation of TEFs requires extensive amounts of testing to quantify toxicity of all chemicals present. Application of tPAH has the ability to estimate the toxicity of a whole mixture but can be highly inaccurate at predicting toxicity if the toxicity of the components are not equal. Quantitative structure activity relationships (QSAR) offer an empirically based method whereby biological reactions to a chemical can be predicted from chemical and physical properties. If mixture toxicity can be accurately predicted, individual oils can be compared during risk assessment based on predicted toxicity and the predicted ecological effects.

QSARs encompass a variety of techniques for predicting parameters of untested compounds based on their structural similarity to chemicals with known activities and properties. This method draws relationships between aspects of chemical structure and an activity or property
imparted by that structure. QSAR model development is dependent on accurate and reliable experimental data. Models are generated using a training set and tested for precision using a validation set; chemicals for both groups are selected from a common group with similar mechanism of toxicity (Leonard and Roy, 2006). Many different parameters can be used for comparison including; electrophilicity, hydrogen bonding, molecular fragments as well as physical-chemical properties such as log P. Typical approaches include the analog approach whereby toxicity is extrapolated from a compound to a metabolite or transformation product, the chemical category approach, which groups chemicals via physical-chemical descriptors, as well as computerized QSAR models which identify active and inactive chemicals based on presence and absence of specific structural features.

A further level of complexity is added as these models are extended to predict mixture toxicity where application of a single chemical toxicity model is likely to be misleading. Primarily this is because chemical parameters change when present in mixtures; solubility limits can decrease and toxic mechanisms can interact and result in additive, synergistic or inhibitory effects (Altenburger et al., 2003). Current methods are effective at deriving basic understandings of relevant interactions and molecular mechanisms but cannot accurately predict mixture toxicity (Hermens et al., 2011). A toxicity model derived from a QSAR for PAH offers the ability to compare individual oils based on PAH composition and predicted toxicity. A comprehensive chemical profile of various PAH, both alkylated and non-alkylated, would help improve our understanding of complex PAH mixtures and the risk they pose to ecologically sensitive areas.

In this study, the chronic toxicity of the 4 ringed PAH benz[a]anthracene (B[a]A) and the alkylated congener 7-methylbenz[a]anthracene (7-MeB[a]A) were evaluated, the relationship between alkylation and various physical-chemical properties was investigated, and the PCD
method was further characterized. Seventeen-day chronic toxicity assays were performed using ELS of Japanese medaka to generate the toxicity endpoint Median Effective Concentration (EC50) for sub lethal responses. Toxicity endpoints were calculated from dose-response relationships between test chemical and the prevalence of BSD. Experimental solubility limits were compared with existing data sets, as were several PCD parameters. The film partition coefficient (log $K_{fs}$) was calculated and describes the ability of test chemicals to partition from the prepared film to the exposure solution, time to steady state (tss) describes the amount of time required for 95% of final equilibrium concentration to be reached, film: solution partitioning rate ($k$) describes the ability of PDMS films to dynamically compensate for chemical loss, and the ability of films to compensate for chemical loss over time. Fluorescence spectrometry was used to determine measured concentrations. This research will further contribute to the development of QSARs while expanding our understanding of the toxicity of complex mixtures of PAH.
**Material and methods**

*Experimental design:*

The PCD exposure method for hydrophobic substances was used to assess the chronic toxicity of B[a]A and the alkylated congener 7-MeB[a]A to ELS of Japanese medaka. Embryos were exposed to nominal film loadings ranging from 3.96E-05 to 3.96E-02 mol/L as well as to a negative control with no chemical added (0 mol/L). Measured concentrations in exposure solutions ranged from 0 to 4.34E-08 mol/L and 0 to 1.34E-08 mol/L for B[a]A and 7-MeB[a]A respectively. Medaka were exposed from two hours post fertilisation until hatch, ranging from 9 to 17 days.

The PDMS films were characterized by log $K_{m}$, $k$, tss, and their ability to deliver exposure concentrations up to the solubility limit of test chemical. These values were compared with literature values and with existing data. Fluorescence spectroscopy was used for chemical analysis and emission/excitation wavelengths optimized for each chemical. The EC50s were determined from the prevalence of BSD and compared with various physical-chemical parameters.

**Chemicals and reagents**

Test compounds B[a]A and 7-MeB[a]A were purchased from Sigma-Aldrich (St. Louis, MO, USA) at the highest purity grade available. Aquarium sealant (PDMS) was purchased from Marineland (Blacksburg, Va, USA). Embryo rearing solution (ERS) (1 ml of 10 % NaCl, 1 ml of 0.3 % KCl, 1 ml of 0.4 % CaCl$_2$•2H$_2$O, 1 ml of 1.63 % MgSO$_4$•7H$_2$O, and 95 ml of double-distilled water) was prepared in-house with standard reagents at their best purity available.
Double-distilled water was prepared in-lab. HPLC-grade ethanol, acetone, and hexane were purchased from Fisher Scientific (Ottawa, ON, Canada).

**Medaka culture and egg collection**

Medaka culture conditions followed Denny *et al.* (1991) under the Queen’s University Animal Care protocol HODSON 2011-038-Or. The culture was kept between 25 and 27 °C with a 18hrLight: 6hrDark photoperiod. Medaka were fed brine shrimp (*Artemia sp.*) daily to ensure continuous oogenesis, and eggs were fertilized by cohabitating males kept at a Female: Male ratio of 3:2. Embryos were collected the same day as exposure and separated based on fertilisation success following a modified version of Benoit *et al.* (1991). A 0.1 percent MS-222 (Tricaine methanesulfonate) was used for anaesthetising fish during observation with a dissecting microscope (Yamamato, 1975).

**Solvent selection and PTI characterisation**

Before films were prepared, appropriate solvents were chosen to deliver chemicals for the various experiments. The solubility of test chemicals was first tested in hexane and various hexane: dichloromethane (DCM) mixtures because the preparation of PDMS films required the delivery of each chemical in hexane (Appendix A). Appropriate solvents for preparing standards were also identified using EtOH and EtOH: Acetone mixtures for both B[a]A and 7-MeB[a]A. Using a dilution series, 4.13E-11- 4.13E-07 mol/L, standards were utilized to characterize test chemicals by the PTI method as described below; optimal emission and excitation wavelengths were determined and individual spectra were characterized using 4.13E-07 mol/L standard. The effect of different solvent: solvent dilutions were also compared to minimize signal interference at lower concentrations.
Preparation assay

Exposure vials were prepared following a modified version of Brown *et al.* (2001, 2003). Using this method, a solution of 6 mg/mL (PDMS dissolved in hexane) was prepared and loaded with a series of PAH concentrations (Appendix B); negative controls were prepared in the same way with no PAH added. Positive controls were not prepared for each exposure, but the method was validated using a known toxicant (retene) and the expected signs of toxicity observed at measured concentrations similar to that of previous work by Turcotte *et al.*, 2011. Solutions were deposited into 20 mL scintillation vials held at a 45° angle (Appendix C). Vials were left overnight to facilitate hexane evaporation and the residual film cured at room temperature. An aliquot of 15 mL ERS was added to establish equilibrium between loaded film and exposure solution after which newly-fertilized medaka embryos were added to initiate the test.

Chemical analysis

Samples were analyzed with a fluorescence spectrophotometer (Quanta Master QM1: Photon Technology International). Emission wavelengths were scanned at an excitation wavelength of 343 nm to find the optimal range for data collection (Appendix D). Each sample was mechanically-mixed using a vortex and sonicated for 3 minutes prior to analysis. Concentrations were derived using a calibration curve consisting of at least five standards in 50:50 ERS: EtOH (or EtOH: acetone) depending on the chemical. A linear regression was calculated using the ascending portion of the standard curve. Statistics (linear regression, $R^2$, normality test) were performed with $p=0.05$ (Microsoft Excel 2007). Blanks were run periodically during analysis to confirm that cuvettes and reagents were uncontaminated. Limits of detection were calculated for both test chemicals (Appendix E) using equation 1 (Appendix E).
**Solubility limit estimation and time to equilibrium**

Exposure vials were prepared as described above. Films were loaded with increasing amounts of test chemical, allowed to cure over night, and 15 mL ERS added. Vials were placed on an orbital shaker at 700-900 rpm for a specified time determined in time-to-equilibrium experiments, to facilitate equilibration of film and ERS. Samples were preserved 50:50 (v:v) in 20% acetone: EtOH and chemically analysed within two weeks. Solubility limits were inferred when the slopes of “film loading” vs. “measured concentration” equalled zero. Inferred solubility limits were compared with estimated values using the software program ALOGPS 2.1 from the Virtual Computational Chemistry Laboratory (VCCLAB), as well as literature values where available. These data were used to select an appropriate range of film loadings to deliver a gradient of exposure concentrations at and below the water solubility limit of each test chemical. The partitioning rates of test chemicals from prepared films to exposure solutions were measured by repeated sampling over time, one sample per vial per time (Appendix F). Each experiment was completed with duplicate vials per sampling time. The log $K_{fs}$ was calculated from the slope of the plot of “film concentration” vs. “measured concentration”. Values for $k$ and $t_{ss}$ were calculated using equations 1 and 2 (Appendix G) where $C_t$ and $C_e$ were measured concentrations at time $t$ and equilibrium, respectively.

**Bioassay procedure**

Following film curing, 15 mL of ERS was added to prepared vials and placed on an orbital shaker at 700-900 rpm for 24-36 hrs to facilitate equilibration of the test chemical between film and ERS. Two fertilized medaka eggs were added to each test vial for a 17-day bioassay (Appendix H). The vials were kept at a 45° angle so that eggs were never in contact with the
PDMS film (Appendix C). Room and water temperatures were monitored daily and the bioassay maintained at 27 ± 1°C as suggested by Benoit et al. (1991). Exposures were completed in 24 hrs darkness except when vials were checked daily for approximately one hour. Exposure vials were wrapped in tinfoil to minimize light exposure and treatments were assigned a number randomly to eliminate bias. Vials were checked daily for hatching success, unfertilized and dead eggs were removed, and vials were opened and agitated to ensure adequate dissolved oxygen concentrations. Exposure concentrations were monitored throughout each test to characterize exposure concentrations and to verify that the exposure solutions were at steady state. Test solutions were sampled and diluted 50:50 (v:v) in 20% Acetone: EtOH.

**Bioassay Observation and EC50 determination**

Exposure vials were checked daily and embryos observed for hatching success, mortality and morphological abnormalities. The prevalence of BSD was scored based on presence (0-1) and severity (0-3) of sign where higher values indicate higher severity. Pericardial (0-3) and yolk sac (0-3) edema were identified as enlargement of the pericardial and yolk sac respectively caused by fluid accumulation. Presence/ absence of swim bladder (0-1), craniofacial malformations (0-1), fin rot (0-1) and hemorrhaging (0-1) were assessed. Spinal deformities (0-1) were identified by curvature of the vertebral column. Tube heart (0-2) was also assessed as elongation of the heart. The maximum BSD score was the sum of all maximum scores for each sign (13) while mortality with signs of BSD was assessed as 13.5. Mortality did not include unfertilized embryos and was assessed after Day 2 of exposure. Scores for each fish were summed across treatments and averaged by the number of fish per treatment to determine the overall BSD score per treatment. The EC50s for percent embryos presenting signs of BSD were calculated using a sigmoidal dose-response equation expressed as;
\[ Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1 + 10^{\left(\text{LogEC50} - \text{X}\right) \cdot \text{Hill Slope}})} \], (GraphPad Prism 6, freeware version; GraphPad software)

and compared with EC50 values estimated using a probit regression model (U.S EPA Environmental Research Lab, Duluth, Minnesota) and a logistic regression model using measured concentrations for calculations. Negative controls were prepared with all exposures (0 mol/L).

**Correlation Endpoints and chemical parameters**

Calculated EC50 values were compared with log \( K_{fs} \) and estimated log P (ALOGPS 2.1). These values were also compared with data from other PAH tested using similar exposure methods, and compared with values predicted by various models.

**Statistics**

Statistical analysis was conducted with Microsoft Excel, Probit Software, and Graph Pad Prism (GraphPad Software, Inc. Version 6). Calculations of EC50 for embryo exposures were conducted using Probit analysis/software. EC50 is the concentration of a chemical causing 50% of the maximal effect for continuous variables (e.g. BSD index) and the concentration causing a response of the median fish for binomial data.
Results

Characterization PCD method for test chemicals

Preloaded PDMS films delivered measured exposure concentrations at and below the solubility limit of both test chemicals. The experimental solubility limit of B[a]A (Figure 1) agreed well with literature values but overestimated solubility when compared with predicted solubility (ALOGPS 2.1) (Table 1). The experimental solubility limit of 7-MeB[a]A (figure 2) overestimated predicted solubility by 28%; no literature values were available for comparison. Measured concentrations increased with film concentration. A gradient of film loadings were determined which provided reliable exposure concentrations for both chemicals tested.
Figure 1. Measured concentration benz[a]anthracene after, equilibration plotted as a function of the PDMS film concentration at 27°C. The dashed lines represent the solubility limit derived from the literature and predicted. Symbols represent average concentrations (n=13), ± SEM. Log $k_{fs}$ was determined by calculating the slope of the linear part of the curve before plateau was reached (film loadings 0- 4.2E-03 mol/L). The curve was drawn by hand to aid identification of experimental solubility limit which was indicated by the plateau of the curve.
Table 1. Experimental, predicted and literature values for solubility and log P of B[a]A and 7-MeB[a]A. Experimental Solubility limits were calculated from the horizontal portion of the plot “film loading” vs. “measured concentration” and log $k_{fs}$ from the slope of the initial linear part of the curve.

<table>
<thead>
<tr>
<th>Test Chemical</th>
<th>log P (ALOGPs)</th>
<th>Predicted Solubility limit (ALOGPs) (mol/L)</th>
<th>Solubility limit (Henny et al., 1998)(mol/L)</th>
<th>Experimental Solubility limit (mol/L)</th>
<th>Log $k_{fs}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>B[a]A</td>
<td>5.72*</td>
<td>2.03E-08</td>
<td>5.47E-08 (4.82E-08 - 6.13E-08)</td>
<td>5.34E-08 (5.30E-08 - 5.39E-08)</td>
<td>6.70</td>
</tr>
<tr>
<td>7-MeB[a]A</td>
<td>6.11*</td>
<td>1.13E-08</td>
<td>NA</td>
<td>1.45E-08 (1.44E-08 - 1.45E-08)</td>
<td>7.39</td>
</tr>
</tbody>
</table>

* [http://www.vcclab.org/lab/alogps/start.html](http://www.vcclab.org/lab/alogps/start.html)
Figure 2. Measured concentration of 7-methylbenz[a]anthracene after equilibration, plotted as a function of the PDMS film concentration at 27°C. The dashed line represents the predicted solubility limit as estimated from ALOGPS 2.1. Symbols represent average concentrations (n=39), ± SEM. Log $k_f$ was determined by calculating the slope of the initial linear part of the curve before plateau was reached (film loadings 0-3.96E-04 mol/L). The curve was drawn by hand to aid identification of experimental solubility limit, which was indicated by the plateau of the curve.
**Time to Equilibrium (tss)**

The experimental time to equilibrium of both test chemicals was longer than originally expected, requiring between 12 and 24 hrs to fully equilibrate between prepared film and exposure solution (Figures 3, 4). Of interest was the increase in concentration from times 0 to 720 minutes (Figures 3, 4). Measured B[a]A concentrations did not change noticeably until they increased rapidly from time 40 to 320 minutes followed by a subsequent decrease in rate as concentrations approached the solubility limit. Measured 7-MeB[a]A concentrations did not begin to increase until 180 minutes, followed by a rapid increase to time 720 minutes and subsequent decrease when the solubility limit was approached between 720 and 1440 minutes (24 hours). The $k$ and $tss$ values (Table 2) were calculated using equations 1 and 2 (Appendix F). Calculated $tss$ values do not m
Figure 3. Release of benz[a]anthracene from preloaded PDMS films in glass 20mL scintillation vials on orbital shaker (600 – 900 rpm) (T=20°C). Symbols represent averaged values using film loadings 4.20E-03 and 8.41E-03 mol/L (n=6 ± SD). The curve is drawn by hand to aid identification concentration plateau. The estimated solubility limit is superimposed as a dashed line.
Figure 4. Release of 7-methylbenz[a]anthracene from preloaded PDMS films in glass 20mL scintillation vials on orbital shaker (600 – 900 rpm) (T=20°C). Symbols represent averaged values using film loadings 3.96E-03 and 3.96E-02 mol/L (n=6 ± SD). Curve is drawn by hand to aid identification of concentration plateau. The estimated solubility limit is superimposed as a dashed line.

Table 2. Calculated values for film: solution partition coefficient (k) and time to steady state (tss) using data from figure 4 and 5 in equations 1 and 2 (Appendix G) for test chemicals benz[a]anthracene and 7-methylbenz[a]anthracene.

<table>
<thead>
<tr>
<th>Test Chemical</th>
<th>k (mol/L/minute)</th>
<th>tss (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B[a]A</td>
<td>0.0076</td>
<td>396</td>
</tr>
<tr>
<td>7-MeB[a]A</td>
<td>0.0046</td>
<td>658</td>
</tr>
</tbody>
</table>
Performance of films over time

Preloaded films provided relatively stable exposure concentrations throughout the 17-day toxicity test. Mean measured concentrations varied significantly (p=0.05) between day 1 and 17 of exposure for B[a]A as day 17 concentrations were significantly higher (figure 5). At the solubility limit, measured concentrations varied by little more than 15%. Differences were more pronounced for the lower four concentrations where increases of 2 and 3 times the original concentration were found. Measured concentrations in vials with embryos added were significantly different from vials with no embryos added. When plotted against expected concentration, mean exposure concentrations before hatch were consistently lower than those measured after hatch (day 17) (Figure 6). No significant difference (p=0.05) was found between measured concentrations on Day 1 and 8 for vials with embryos added, and no embryos added, and were combined to represent exposure concentrations. No significant differences were found between mean measured concentrations for 7-MeB[a]A loaded films (n=65) with time (Figure 7). Films compensated for chemical loss and provided stable exposure concentrations throughout the 17- day toxicity test, but concentrations in vials with no embryos added appear to decrease significantly after 17 days although no significant difference was found in measured concentration over time.
Figure 5. Measured exposure concentration benz[a]anthracene in vials without eggs (n=21), in vials with eggs (n=70) and all vials combined (n=91). Each symbol represents an average on that day ± SEM. All exposure vials were included and concentrations normalized. The dashed line represents hatch day and the solid line represents expected concentration calculated as the average measured concentration of Day 1 and 8 (n=182) (p=0.05).
Figure 6. Measured concentration benz[a]anthracene in vials containing medaka eggs are compared before and after hatch. Solid line is used to indicate agreement between measured and expected values (1:1). The solubility limit derived from literature is superimposed as a dashed line.
Figure 7. Measured exposure concentration of 7-methylbenz[a]anthracene in vials without eggs (n=15), in vials with eggs (n=50) and all vials combined (n=65). Each symbol represents the average measured concentration on that day ± SEM. All exposure vials were included and concentrations normalized. The dashed line represents hatch day and the solid line represents expected concentrations calculated as the average measured concentration of all sampling times (n=195) (p=0.05).

Toxicity

Tested chemicals did not cause acute lethality or high levels mortality throughout toxicity tests (Appendix I). Both test chemicals were toxic to exposed medaka at tested concentrations. A positive dose- response relationship was observed for both chemicals. In all treatments for both test chemicals, the percent of medaka presenting signs of BSD were above controls. The alkylated congener 7-MeB[a]A was more toxic than B[a]A as indicated by the lower calculated EC50 (Table 3). The EC50 derived using the logistic approach was considered correct due to the
smaller confidence limits associated with the data compared to the other approaches used (Table 3). A similar positive dose- response relationship was found for both the BSD score and the BSD index with exposure to B[a]A and 7-MeB[a]A (Appendix J). The response of medaka to B[a]A exposure was most noticeable at the higher exposure concentrations. The % affected increased to over 65% (Figure 8), similar to the increase in the BSD index. The Response to 7-MeB[a]A was similar but characterized by an increased response at lower test concentrations (Figure 9). The most prevalent signs of BSD observed for B[a]A included; pericardial edema, craniofacial malformations, spinal deformities and absence of swim bladder. Prevalent signs of BSD observed for 7-MeB[a]A included; pericardial edema, absence swim bladder, craniofacial malformations and body hemorrhaging. Both tested compounds presented similar toxicity as BSD, and had similar dose-response slopes (Table 4).
Table 3. The EC50 of benz[a]anthracene and 7-methylbenz[a]anthracene for percent medaka affected calculated using three software programs. All end-points were generated using measured concentrations, data for benz[a]anthracene are from Day 1 and 8 as no significant statistical difference existed (p=0.05) (n=26), while data used for 7-methylbenz[a]anthracene were generated by combining data from all sampling times as no significant difference was found in measured concentration with time (p= 0.05) (n=39).

<table>
<thead>
<tr>
<th>Test Chemical</th>
<th>PCD logistic EC50 (95% CI) (mol/L)</th>
<th>PCD probit EC50 (95% CI)(mol/L)</th>
<th>PCD GraphPad EC50 (95%CI)(mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B[a]A</td>
<td>2.51E-08 (1.77E-08 - 4.24E-08)</td>
<td>3.33E-08 (2.56E-08 - 6.66E-08)</td>
<td>2.65E-08 (1.53E-08 - 4.60E-08)</td>
</tr>
<tr>
<td>7-MeB[a]A</td>
<td>9.41E-09 (2.68E-09 - 1.34E-08)</td>
<td>1.15E-08 (8.09E-09 - 6.27E-08)</td>
<td>6.15E-09 (2.39E-09 - 1.82E-08)</td>
</tr>
</tbody>
</table>

Table 4. Slope of the linear portion of the dose response curves (hill slope) for benz[a]anthracene and 7-methylbenz[a]anthracene as calculated by GraphPad with 95% confidence limits (Figure 8 & 9).

<table>
<thead>
<tr>
<th>Test Chemical</th>
<th>Hill Slope (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B[a]A</td>
<td>0.70 (0.2398- 1.862)</td>
</tr>
<tr>
<td>7-MeB[a]A</td>
<td>0.60 (0.037- 1.2)</td>
</tr>
</tbody>
</table>
Figure 8. Frequency of abnormal medaka embryos after a 17-day bioassay to varying concentrations of benz[a]anthracene. Measured concentrations derived from Day 1 and 8 of exposure (n=26). The sigmoidal dose-response curve was used to calculate EC50 values. The dashed line represents the EC50 determined visually and the solid line the experimental solubility limit.
Figure 9. Frequency of abnormal medaka embryos after a 17-day bioassay to varying concentrations of 7-methylbenz[a]anthracene. Measured concentrations derived from combined data of all sampling times (n=39). The sigmoidal dose-response curve was used to calculate EC50 values. The dashed line represents the EC50 determined visually and the solid line the experimental solubility limit. The outlined data point has been highlighted to emphasize departure from expected trend of increasing response with measured concentration.
Correlation of EC50s with physical-chemical parameters

The EC50 increased with alkylation, as did logP, logkfs, tss and AhR potency. The k decreased with alkylation, which is consistent with an increase in logP and tss. Calculated log kfs values for test chemicals were consistently higher than found in previous work with other PAH (Brown et al., 2001). The tss and k for test chemicals were longer than found previously for 3-5 ringed PAH by Brown et al., 2001 but compared well with work from Chang et al., 2000 on B[a]A. Toxicity increased with alkylation as found with phenanthrenes by Turcotte et al., 2011.
Discussion

Characterization PCD method for test chemicals

The PCD method of exposure delivered stable concentrations for both test chemicals at and below their solubility limit. Experimental solubility limits found for B[a]A agreed with values found elsewhere (Henny et al., 1998), while published data for 7-MeB[a]A were unavailable. Compared to values predicted from ALOGPS 2.1, measured solubility limits were consistently higher. The ALOGPs 2.1 underestimated the solubility of B[a]A by almost 3 times and 7-MeB[a]A by 28%. These differences in solubility may be due to analytic error at low measured concentrations, the presence of test chemical crystals from overloaded films which would bias measured concentrations, or the inherent difficulty in developing modeling programs. There is also the possibility that the experimental solubility limits were underestimated due to the salting out effect of the ERS solution used throughout these experiments (Chai, 2004) and absorption of chemical onto the glass exposure vials. A false positive may have occurred if film concentrations were not tested at a high enough loading subsequently underestimating solubility.

Calculated log \( K_{fs} \) values did not compare well with previous findings for 3-5 ringed PAH and were consistently higher (Brown et al., 2001). Differences found may be due to different diffusive properties (modelled by log P) affecting the ability of chemicals to partition from the PDMS film. Test chemicals had higher log P values than all PAH tested (Brown et al., 2001), with the exception of Benzo[a]pyrene, which supports the conclusion that higher log P correlates with higher \( K_{fs} \) values. Turcotte, 2008 found a linear relationship between log P and log \( K_{fs} \) (Appendix K) for alkylated derivatives of phenanthrene. When this relationship was applied to the test chemicals, calculated log \( K_{fs} \) values were consistently lower than experimental. A
separate linear relationship may exist to describe alkylated benz[a]anthracenes. If these properties do vary linearly, as found with Phen and alkyl- phenanthrenes, untested alkyl PAH film loadings can be predicted from log P. This would help identify appropriate ranges for film loadings, decreasing the number of preliminary experiments and reducing overall costs.

*Time to equilibrium;*

Time to equilibrium describes the ability of films to dynamically compensate for chemical loss. Previous research has found equilibration times ranging from 45 minutes to 2 hours for the 3-ringed PAH anthracene and Phen respectively. Experiments completed with B[a]A and 7-MeB[a]A have indicated slower partitioning from the highly cross linked PDMS polymer as tss and k were both longer than found with other 3-5 ringed PAH (Brown *et al.*, 2001). Interestingly calculated tss values did not compare well with figures 3 &4 after a visual inspection, where tss appears to be attained at times approaching 1500 minutes for B[a]A and 2500 minutes for 7-MeB[a]A. Chang *et al.*, 2000, found B[a]A to have an equilibration time, from a preloaded film, of over 12 hours (720 minutes), while the larger 5-6 ringed PAH did not fully equilibrate during a 60 hour time course sampling (3600 minutes). Equilibrium times from this study for B[a]A are closer to those of Chang *et al.*, (2000). Time to steady state followed a similar trend and was much longer than previously calculated for Phen and was consistent with an increase in time to equilibrium. Of note was the initial lag time and variation seen in concentration as time initially increased. Concentrations did not begin increasing during time to equilibrium experiments until after an hour had elapsed (Figure 3, 4). The apparent lapse in concentration change may be due to analytical error at these low concentrations as lower concentrations were below detection limit (Appendix E) or the apparent slow partitioning of 4-6 ringed PAH from PDMS films, indicating a resistance to diffusion. This information may indicate a limit for
PDMS films to effectively deliver higher molecular weight compounds, as the ability to generate reliable exposure concentrations is compromised by the films’ capacity to compensate rapidly for chemical loss. This increase in equilibration time may be explained by their high molecular mass and lower diffusion coefficients. Also of interest is the difference seen between measured concentration determined during time to equilibrium studies (T=20°C) and bioassays (T=27°C). An increase in solubility can be seen with temperature, this is supported by similar findings by Ai (1997) where diffusive properties of high molecular weight PAH increased with temperature.

*Stability through time*

The PCD method of exposure has been used previously to deliver stable exposure concentrations throughout chronic toxicity assays (Turcotte *et al.*, 2011). In this study, films delivered exposure concentrations over 17 days which declined by less than 33% suggesting that partitioning rate of test chemicals used was sufficient to provide stable exposure concentrations throughout the bioassay. A general increase in concentration was found for B[a]A at both sampling times throughout exposure, which has been seen with other PAH by Brown *et al.*, 2003. Films prepared with 7-MeB[a]A were able to effectively compensate for loss as no statistical difference was found in mean exposure concentration among all sampling times. Concentrations higher than the solubility limit may be attributed to the presence of dissolved hatching proteins or egg detritus which adsorb PAH from exposure solution, resulting in waterborne PAH that is not freely dissolved but is still measurable by fluorescence. A mechanism for chemical loss would be introduced after hatch, when a transition occurs in the kinetic uptake of the test chemical. Before hatch, uptake is limited by chorion permeability whereas upon emergence, chemicals can be taken up more quickly by medaka because embryonic membranes are more permeable than the
chorion. The more rapid uptake would change the water: film equilibrium if partitioning rates from the film were not sufficient to compensate.

**EC50 and relationship with test chemical parameters**

Benz[a]anthracene is one of 16 PAH listed as a priority pollutant in the Clean Air Act (US-EPA, 1990). Through morpholino knockdown of the AhR receptor, it was shown that B[a]A toxicity is partly mediated through the AhR pathway, independent of CYP1A enzyme metabolism (Incardona et al., 2006). The same study found B[a]A to be cardiotoxic to fish embryos at nominal concentrations above its solubility limit. Toxicity data for 7-MeB[a]A are limited, but studies with rat liver homogenates suggest that despite differences in biological activity among alkylated B[a]As, metabolic pathways are similar (Dower & Yang, 1975).

The results of the present study agree with previous research on the effect of PAH alkylation on toxicity and indicate similar modes of toxicity as TCDD. Alkylation increases the toxicity of many PAH (Turcotte et al., 2011), especially when producing a compound with distal substitution similar in shape to that of TCDD (Billiard et al., 2002). Similarly I found that alkylation increased the toxicity of B[a]A, as 7-MeB[a]A was three times more toxic. The ability of both chemicals to induce similar signs of BSD, increases seen in toxicity with alkylation at a distal position, and similar dose response slopes, indicate that the mechanism of toxicity for both chemicals is similar to that of TCDD. Although B[a]A does not induce CYP1a enzymes (Incardona et al., 2006), toxicity is AhR dependent. Following a similar trend to toxicity, AhR potency was also found to increase with alkylation (Barron et al., 2004). If AhR potency is indicative of B[a]A toxicity, this may represent an appropriate model for the prediction of 4-ringed PAH toxicity. The narcosis model of toxicity suggests a relationship between toxicity, log
Kow and rate of accumulation. With an increased log Kow, toxicity is expected to increase while rate of uptake is likely to decrease (McCarty et al., 1985). This mechanism of toxicity is likely to contribute to the chronic toxicity of these chemicals as indicated by the lack of acute mortality and high log kow.

*Ecological Considerations*

Alkylated benz[a]anthracenes are toxic to the ELS of fish species at ecologically relevant concentrations, i.e. at concentrations below their solubility limit. As known constituents of oil, these PAH are likely to contribute to PAH mixture toxicity. The toxic effects of these chemicals to fully-developed fish are unknown but their ability to induce symptoms of the syndrome BSD in the ELS of Japanese medaka indicates risk to both health and reproduction of exposed fish populations. These chemicals are toxic to fish and may represent a significant threat to aquatic life if remaining in the environment for prolonged periods of time following an oil spill.

*Research considerations*

If this project were repeated, there are some issues that should be addressed. In PCD characterization experiments, different surface area: volume ratios of PDMS films could be used to investigate their effect on the diffusive properties of high molecular weight PAH. Various surface area: volume ratios should be tested using a time coursed sampling procedure to determine these effects. Measured concentrations should be analyzed using fluorescence spectroscopy and a range of film loadings similar to that used during chronic toxicity assays of this study would be ideal for this water chemistry experiment. If diffusion is found to increase with higher surface area: volume ratios, this may represent a method for ameliorating the ability of films to compensate rapidly for chemical loss.
Another limitation of this study was that chemical uptake by medaka was not measured. Toxicity was assumed to be the sum of chemical exposure throughout the toxicity assay (9 to 17 days depending on hatch date); yet previous research has shown a correlation between log kow and kinetic uptake across the chorion as a limitation to toxicity. An experiment designed to compare tissue concentrations between a group of exposed embryos before hatch and a separate group after hatch could be performed to test this hypothesis. Embryos and hatchlings could be ground and analyzed via fluorescence spectroscopy for a rough measurement of tissue concentrations and therefore chemical uptake. A range of film loadings similar to that used during the chronic toxicity assay would be ideal for this bioaccumulation experiment. An experiment of this design would also allow validation of models which predict critical body burden and toxicity from Kow and waterborne concentrations.

Future directions for this study should include performing chronic toxicity assays using other alkylated benz[a]anthracenes to further test how toxicity correlates with log P and AhR potency. This would further our understanding of the effect alkylation has on toxicity of benz[a]anthracenes and contribute to the development of QSAR for these alkylated compounds. Chronic toxicity assays could also be extended to include other 4-ringed PAH and their alkylated congeners to further develop predictive models and our understanding of how PAH toxicity is mediated by size and alkylation.
Literature Cited:


Summary

2. The experimental solubility limit for 7-MeB[a]A is the first experimentally reported.
3. Benz[a]anthracene and alkyl benz[a]anthracene were toxic to the ELS Japanese medaka below their solubility limits, exhibiting a positive dose response relationship between exposure concentration and sub-lethal effects.
4. Alkylation increased the toxicity of B[a]A.
5. Similar dose-response slopes and signs of blue sac disease indicated mechanisms of toxicity similar to that of TCDD for both test chemicals.
6. Toxicity increased with log P and AhR potency, providing two possible models for the prediction of alkyl- benz[a]anthracene toxicity.
Appendix A:

Table. Solvent selection for test chemicals, PDMS film preparation required delivery of test chemicals in hexane, hexane:dichloromethane. Standard preparation and preserving solvent utilized EtOH or Acetone: EtOH.

<table>
<thead>
<tr>
<th>Test Chemical</th>
<th>Solvent dilution used for PDMS film preparation (mg/mL)</th>
<th>Solvent dilution used for standard preparation (mg/mL)</th>
</tr>
</thead>
</table>

Appendix B:

Table. Dilution series used for PCD film preparation for benz[a]anthracene and 7-methylbenz[a]anthracene.

<table>
<thead>
<tr>
<th>Film series</th>
<th>Concentration B[a]A film (mol/L)</th>
<th>Concentration 7-Meb[a]A film (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.31E-04</td>
<td>3.96E-05</td>
</tr>
<tr>
<td>2</td>
<td>2.63E-04</td>
<td>1.98E-04</td>
</tr>
<tr>
<td>3</td>
<td>5.26E-04</td>
<td>3.96E-04</td>
</tr>
<tr>
<td>4</td>
<td>1.05E-03</td>
<td>3.96E-03</td>
</tr>
<tr>
<td>5</td>
<td>2.10E-03</td>
<td>3.96E-02</td>
</tr>
<tr>
<td>6</td>
<td>4.20E-03</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>8.41E-03</td>
<td>N/A</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Appendix C:

Figure 1. Partition Controlled Delivery exposure method.

Appendix D:

Table. Optimized Emission and Excitation wavelengths used for benz[a]anthracene and 7-methylbenz[a]anthracene. Emission and excitation scanning used a step size of 2nm.

<table>
<thead>
<tr>
<th>Test Chemical</th>
<th>Emission Wavelength (nm)</th>
<th>Excitation Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B[a]A</td>
<td>353-483</td>
<td>343</td>
</tr>
<tr>
<td>7-MeB[a]A</td>
<td>352-482</td>
<td>342</td>
</tr>
</tbody>
</table>

Appendix E:

Table: Calculated values for detection limit of test chemicals benz[a]anthracene and 7-methylbenz[a]anthracene using equation 1.

<table>
<thead>
<tr>
<th></th>
<th>B[a]A (mol/L)</th>
<th>7-MeB[a]A (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection Limit</td>
<td>2.82E-9</td>
<td>1.90E-9</td>
</tr>
</tbody>
</table>
Equation 1:

\[
\text{Limit of Detection} = 3.3 \times \left( \frac{\text{Standard deviation of the response}}{\text{Slope of calibration curve}} \right)
\]

Appendix F:

Table. Sampling procedure for time to equilibrium experiment for benz[a]anthracene and 7-methylbenz[a]anthracene. Individual vials \(n=2\) were used at each sampling time.

<table>
<thead>
<tr>
<th>Sampling</th>
<th>Sampling Time (minutes); B[a]A</th>
<th>Sampling Time (minutes); 7-MeB[a]A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>22.5</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>180</td>
</tr>
<tr>
<td>5</td>
<td>160</td>
<td>360</td>
</tr>
<tr>
<td>6</td>
<td>320</td>
<td>720</td>
</tr>
<tr>
<td>7</td>
<td>640</td>
<td>1440</td>
</tr>
<tr>
<td>8</td>
<td>1440</td>
<td>2880</td>
</tr>
</tbody>
</table>
Appendix G:


Equation 1: \( C_t = C_e \times (1-e^{-kt}) \)

\( C_t \): Measured concentration at time \( t \)

\( C_e \): Measured concentration at equilibrium

\( k \): Film release rate constant (\( C_t \sim 0.95 \ C_e \))

\( t \): time (minutes)

Equation 2: \( t_{ss} = \frac{3}{k} \)

\( t_{ss} \): Time to steady state

\( k \): Film release rate constant

Appendix H:

Table. Experimental design for benz[a]anthracene and 7-methylbenz[a]anthracene toxicity tests.

<table>
<thead>
<tr>
<th>Test Chemical</th>
<th>Treatments</th>
<th>Replication</th>
<th>Sampling</th>
</tr>
</thead>
</table>
| B[a]A         | 7 Concentrations + 1 Negative control | 13 vials per treatment (10 with embryos, 3 no embryos added) | 1. Before embryo addition  
               |                                |                                                       | 2. Day 8 (hatch)  
               |                                |                                                       | 3. After both embryos were removed from the vial |
| 7-MeB[a]A     | 5 Concentrations + 1 Negative Control | 13 vials per treatment (10 with embryos, 3 no embryos added) | 1. Before embryo addition  
               |                                |                                                       | 2. Day 8 (hatch)  
               |                                |                                                       | 3. After both embryos were removed from the vial |
Appendix I:

Figure I.1. Percent mortality of exposed medaka plotted as a function of measured concentrations in ERS of benz[a]anthracene. Measured concentration data pooled from Day 1 and 8 sampling during exposure (n=26). Mortality was monitored daily and dead embryos/hatchlings removed following Day 2 of exposure when unfertilized embryos were removed.

Figure I.2. Percent mortality of exposed medaka plotted as a function of measured concentrations in ERS of 7-methylbenz[a]anthracene. Measured concentration data has been pooled from all sampling times (n=39). Mortality was monitored daily and dead embryos/hatchlings removed following Day 2 of exposure when unfertilized embryos were removed.
Table I.1. Percent unfertilized medaka embryos, assessed during Day 1 and 2 of exposure.

<table>
<thead>
<tr>
<th></th>
<th>Benz[a]anthracene (n= 160)</th>
<th>7-methylbenz[a]anthracene (n=120)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfertilized embryos (%)</td>
<td>6.88%</td>
<td>8.33%</td>
</tr>
</tbody>
</table>

Appendix J:

Figure J.1. Measured concentration in ERS of benz[a]anthracene plotted with BSD Score of exposed medaka. Measured concentration samplings on Day 1 and 8 of exposure have been pooled (n=26). The BSD data per treatment was summed and divided by number of medaka per treatment (n=15- 20) using maximum score of 13.5. The dashed line represents positive dose response relationship.
Figure J.2. Measured concentration in ERS of benz[a]anthracene plotted with BSD Index of exposed medaka. Measured concentration samplings on Day 1 and 8 of exposure have been pooled (n=26). The BSD data per treatment was summed and divided by number of medaka per treatment (n=15-20) and normalized using maximum BSD score of 13. The dashed line represents positive dose response relationship.

Figure J.3. Measured concentration in ERS of 7-methylbenz[a]anthracene plotted with BSD Score of exposed medaka. Measured concentration are pooled from all sampling times of exposure (n=39). The BSD data per treatment was summed and divided by number of medaka per treatment (n=15-20) using maximum score of 13.5. The dashed line represents positive dose response relationship.
Figure J.4. Measured concentration in ERS of 7-methylbenz[a]anthracene plotted with BSD Index of exposed medaka. Measured concentrations are pooled from all sampling times (n=39). The BSD data per treatment was summed and divided by number of medaka per treatment (n=15-20) and normalized using maximum BSD score of 13. The dashed line represents positive dose response relationship.

Appendix K:

\[ y = 0.93x - 0.07 \]

\[ R^2 = 0.83 \]

Linear relationship between log Kfs and log P as found by Turcotte, 2008.