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Quantitative structure–activity relationships for chronic toxicity of alkyl-chrysenes and alkyl-benz[a]anthracenes to Japanese medaka embryos (*Oryzias latipes*)

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ABSTRACT

Alkylated polycyclic aromatic hydrocarbons (alkyl-PAHs) are a class of compounds found at significant concentrations in crude oils, and likely the main constituents responsible for the chronic toxicity of oil to fish. Alkyl substituents at different locations on the aromatic rings change the size and shape of PAH molecules, which results in different interactions with tissue receptors and different severities of toxicity. The present study is the first to report the toxicity of several alkylated derivatives of chrysene and benz[a]anthracene to the embryos of Japanese medaka (*Oryzias latipes*) using the partition controlled delivery (PCD) method of exposure. The PCD method maintained the desired exposure concentrations by equilibrium partitioning of hydrophobic test compounds from polydimethylsiloxane (PDMS) films. Test concentrations declined by only 13% over a period of 17 days. Based on the prevalence of signs of blue sac disease (BSD), as expressed by median effective concentrations (EC50s), benz[a]anthracene (B[a]A) was more toxic than chrysene. Alkylation generally increased toxicity, except at position 2 of B[a]A. Alkyl-PAHs substituted in the middle region had a lower EC50 than those substituted at the distal region. Except for B[a]A and 7-methylbenz[a]anthracene (7-MB), estimated EC50 values were higher than their solubility limits, which resulted in limited toxicity within the range of test concentrations. The regression between log EC50s and log K_{ow} values provided a rough estimation of structure–activity relationships for alkyl-PAHs, but K_{ow} alone did not provide a complete explanation of the chronic toxicity of alkyl PAHs.

1. Introduction

The chronic toxicity of petroleum hydrocarbons to early life stages of fish is one of the most severe impacts of marine and freshwater oil spills. The components most closely associated with chronic toxicity are alkylated polycyclic aromatic hydrocarbons (alkyl-PAH) with three or more fused benzene rings and one to four alkyl substituents (Adams et al., 2014; Bornstein et al., 2014; Hodson et al., 2007a). These 3-ring and 4-ring (i.e., tricyclic and tetracyclic) alkyl-PAHs cause a syndrome called blue sac disease (BSD) in early life stages of fish. BSD is a non-contagious and irreversible disease expressed as a variety of signs that include increased activity of CYP1A enzymes (particularly in liver), heart and craniofacial deformities, yolk sac and pericardial edema, hemorrhaging, and mortality before swim up (Bauder et al., 2005; Billiard et al., 1999; Incardona et al., 2011; Marty et al., 1997).

The mechanisms of chronic toxicity vary among different unsubstituted and alkyl substituted PAHs. Toxicity could be mediated through the interaction of PAH with the aryl hydrocarbon receptor (AHR) protein (Billiard et al., 2002; Scott et al., 2011), cytochrome P450 enzymes (Bauder et al., 2005; Hawkins et al., 2002), cardiac receptors (Incardona et al., 2006, 2011), and lipid membranes (narcosis) (Turcotte et al., 2011; Verhaar et al., 1992). Toxicity may also be due to the derivatives of PAH following CYP1A oxygenation (Fallahtafti et al., 2012; Hodson et al., 2007b; Scott and Hodson, 2008), or phototransformation (Oris and Giesy, 1987; Turcotte, 2008).

There are more than 500 possible congeners of C1–C4 alkyl phenanthrenes and they appear more toxic to Japanese

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medaka (*Oryzias latipes*) embryos than unsubstituted phenanthrene (Turcotte et al., 2011). Given that individual alkyl-PAHs, even with similar structures, demonstrate significant variations in embryo toxicity, the development of structure–activity relationships (SARs) based on model compounds could provide important tools for estimating the toxicity of PAH, individually and in mixtures, for ecological risk assessments (Di Toro et al., 2007). Toxicity increases with molecular size up to a limit, beyond which it decreases, likely due to slower uptake or limited aqueous solubility (Brown et al., 2001; Turcotte et al., 2011). Structural features such as the size and position of alkyl substituents on the aromatic core affect the toxicity of alkyl-phenanthrenes; substituents in the distal regions (C-1, 2, or 7) of molecules lead to a higher toxicity than those in the mid-regions (C-9 or 10) (Kiparissis et al., 2001). Turcotte et al. (2011) proposed that the more the structure of alkyl-substituted phenanthrenes resembled retene (7-isopropyl-1-methylphenanthrene), the greater the toxicity to fish. Moreover, the toxicity of alkyl-phenanthrenes may depend on their capacity to undergo oxygenation to *para*-quinones (Fallahtafti et al., 2012), or on their binding affinity for specific protein receptors such as the AHR (Billiard et al., 2002). In current risk assessment models, *n*-octanol acts as a surrogate for cell membrane lipids. For many PAHs, there is a strong relationship between threshold concentrations for chronic embryo toxicity and octanol–water partition coefficients (K_{ow}) (McGrath and Di Toro, 2009), and K_{ow} has been used widely in risk assessments of PAH mixtures (Di Toro and McGrath, 2000; Di Toro et al., 2007; McGrath and Di Toro, 2009;). In general, the chronic toxicity of alkyl-phenanthrenes increases with $\log K_{ow}$ (Turcotte et al., 2011), reflecting enhanced exposure to more hydrophobic PAH, and potential interactions with protein receptors that have a hydrophobic character, such as the AHR (Billiard et al., 2002).

There is limited knowledge on the embryo toxicity of tetracyclic PAHs, especially the alkylated congeners, in part because these compounds are not commercially available and have never been synthesized in testable quantities. A recent comparison of the embryo toxicity of chrysene and B[a]A demonstrated that the latter is more toxic, despite similar chemical structures and K_{ow} values (Incardona et al., 2006). While alkyl derivatives of these compounds were present in fractions of crude oil that caused embryo toxicity (Hodson et al., 2007a), there are few reported measurements of the embryo toxicity of individual alkyl-chrysenes and B[a]As, mainly because most alkyl PAH are difficult to synthesize in pure form (Cai et al., 2004). In standard gas chromatography/mass spectrometry (GC/MS) analyses, chrysene and B[a]A, as well as their alkyl-congeners, are usually reported as a single group. However, if individual compounds show significant differences in toxicity, it is critical to treat these isomeric compounds separately in chemical analysis, and to have accurate SAR models for ecological risk assessments.

A new method of exposure, the partition-controlled delivery (PCD) of compounds from a thin polydimethylsiloxane (PDMS) film to aqueous solutions, has been developed to overcome the problems of testing sparingly soluble (hydrophobic) PAHs (Brown et al., 2001; Kiparissis et al., 2003; Seiler et al., 2014). With PCD, desired concentrations at or below the solubility limit of test compounds are maintained for a prolonged period of time while using only a small amount of test compound. If test compounds are lost from the aqueous solution, the PDMS film can rapidly compensate for the loss by releasing more compounds to maintain a stable concentration for exposure tests. However, the PCD method has not been applied to tests of tetracyclic alkyl-PAHs and its ability to maintain a constant aqueous concentration must be evaluated before toxicity testing.

The present study is the first to measure the chronic toxicity of a series of alkyl-substituted chrysenes and B[a]As to medaka embryos, and the first to report the synthesis of some of these compounds by directed remote metalation (Cai et al., 2004). It is also the first to assess the suitability of a statistical model relating the embryo toxicity of alkylated derivatives of chrysene and B[a]A to K_{ow}. Medaka embryos were exposed to a range of aqueous concentrations of test compounds using PCD, and observed daily to identify abnormalities. The aqueous concentrations of each compound were measured by fluorescence spectrometry and the index of toxicity was the median effective concentration (EC50) for the percentage of affected fish. This study provides a better understanding of the toxicity of these alkyl-PAHs and, more importantly, it suggests that SARs based on K_{ow} should be improved by consideration of the mechanistic interactions of alkyl PAH with cellular receptors.

2. Material and methods

2.1. Chemicals and reagents

Medaka embryos were exposed to chrysene, benz[a]anthracene (B[a]A), 1-methylchrysene (1-MC), 6-methylchrysene (6-MC), 2-methylbenz[a]anthracene (2-MB), 7-methylbenz[a]anthracene (7-MB), and 2,3-dimethylbenz[a]anthracene (2,3-DMB) (Table 1). Synthesis of 1-MC and 6-MC at the U. of Stavanger, Stavanger, NO followed Jørgensen and Joensen (2008) and synthesis of 2-MB and 2,3-DMB at Queen's U., Kingston, ON followed Cai et al. (2004) (details, Section SD-1). Chrysene was purchased from Sigma–Aldrich (Bellefonte, PA, USA), and B[a]A and 7-MB were purchased from Sigma–Aldrich (St. Louis, MO, USA). Unpurified 7-isopropyl-1-methylphenanthrene (retene; >80% purity by high pressure liquid chromatography. Data not shown) was obtained from MP Biomedicals LLC (Illkirch, France) to establish standard operating procedures for different tests, and as a positive control in tests with 2-MB and 2,3-DMB.

Polydimethylsiloxane (PDMS) aquarium-grade sealant 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% $CaCl_2{\cdot}2H_2O$, 1 mL of 1.63% $MgSO_4{\cdot}7H_2O$, 10 μL of methylene blue, and 95 mL of double-distilled water) was prepared with standard reagents at their best purity available. Double-distilled water was prepared using the ELGA PURELAB® Ultra water system (Siemens Water Technologies, Mississauga, ON, Canada). HPLC-grade acetone, hexane, and dichloromethane (DCM) were purchased from Fisher Scientific (Ottawa, ON, Canada). Anhydrous ethanol was obtained from GreenField Ethanol Inc. (Brampton, ON, Canada). Tricaine methane sulfonate (MS-222; Sigma–Aldrich, Oakville, ON) was used as a fish anesthetic during hatchling observation.

2.2. Medaka culture and egg collection

The fish culture was maintained at 25–27 °C with a 16L:8D photoperiod (Kirchen and West, 1999) and the ratio of female to male fish was 3:2 (Denny et al., 1991). Adult medaka were fed brine shrimp nauplii (*Artemia* sp.) 2–3 times a day during the light period to ensure continuous oogenesis. Eggs that were attached to each female's vent by chorionic filaments were collected manually from multiple females. After collection, fertilized eggs (those that were clear and contained double chorionic membranes) were separated from unfertilized eggs using dissecting needles. The newly fertilized embryos were used immediately for toxicity tests.

2.3. Preparation and characterization of the PCD assay

A preloading method was used to prepare PDMS films for the PCD bioassay test vials, modified from Turcotte et al. (2011)