ETHOXYRESORUFIN-O-DEETHYLASE INDUCTION IN TROUT EXPOSED TO MIXTURES OF POLYCYCLIC AROMATIC HYDROCARBONS

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Abstract—This study investigated whether ethoxyresorufin-O-deethylase (EROD) activity in rainbow trout exposed to mixtures of polycyclic aromatic hydrocarbons (PAHs) could be predicted from induction equivalency factors (IEF). The test PAHs were classified into strong and weak inducers on the basis of similar exposure–response curves. Induction equivalency factors of strong inducers, based on benzo[k]fluoranthene (BkF) as the reference compound, ranged from 0.03 to 0.16. Trout exposed to mixtures of strong inducers (2, 4, and 6 equipotent parts) at 0.32-, 1.0-, or 3.2-nM BkF-equivalents showed exposure-dependent increases in EROD activity, consistent with an additive interaction. The extent of activity did not vary greatly among mixtures and single PAHs at a given induction equivalent quantity (IEQ). Induction equivalency factors could not be calculated for weak inducers because the range of induction was too low. Hence, each weak inducer was added to mixtures at concentrations that induced EROD activity fivefold. These mixtures appeared additive because binary and quaternary mixtures caused about 10- and 20-fold induction, respectively. Strong inducers mixed the same way also showed additivity. In contrast, EROD induction by mixtures containing both strong and weak PAHs was 800 to 900% greater than expected, suggesting synergistic interactions. Therefore, if mixtures are composed of PAHs that behave similarly, IEFs may be a suitable approach for assessing risk. However, for mixtures that contain PAHs of differing potency and efficacy, bioassays will be a more reliable measure of risk than IEFs.

Keywords—Polycyclic aromatic hydrocarbons Mixtures Induction equivalency factors CYP1A Fish

INTRODUCTION

Complex mixtures of polycyclic aromatic hydrocarbon (PAH) compounds have been found in waterways polluted with industrial effluents and petrochemical products [1–4], and an estimated 230,000 metric tons enter the aquatic environment every year [5]. Mixtures of PAHs in sediment, oil, creosote, and coal tar include both alkyl-substituted and unsubstituted forms [3,4]. While previous studies have examined mixtures as substances, little research has been conducted on their components and how they interact to produce mixture effects.

PAH toxicity

Polycyclic aromatic hydrocarbons disrupt the development, immunity, reproduction, growth, and survival of aquatic organisms [6–9]. In larval fish, recent studies have demonstrated that PAHs cause blue sac disease [10,11], a syndrome shared with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and other halogenated aromatic hydrocarbons (HAH) [12]. Field and laboratory studies on the Exxon Valdez oil spill have correlated the prevalence of blue sac disease in larval pink salmon to the concentrations of alkyl-substituted (C1–C4) phenanthrenes [10], a finding consistent with the toxicity to larval trout of 7-isopropyl-1-methylbenzanthracene, commonly known as retene [11]. In both cases, the observed toxicity was paralleled by an increased induction of cytochrome P450 (CYP1A) monooxygenases, as characterized by CYP1A protein measurements (immunohistochemistry) [10] or by EROD activity [11].

For various HAH, toxicity to early life stages of fish parallels potency for CYP1A induction, which is consistent with a possible role of CYP1A gene activation or enzyme activity in HAH toxicity [13]. One possible mechanism of toxicity associated with increased CYP1A activity is the formation of oxiradicals or reactive metabolites. Oxidative damage results when oxiradical concentrations exceed the capacity of an organism’s endogenous antioxidant defense system. Oxiradicals may react with DNA, proteins, and membrane lipids, causing oxidative stress and cell death [14]. Reactive metabolites are often electrophilic and may also damage DNA, proteins, and lipids, and many of the symptoms of HAH and retene toxicity to larval fish (edema, hemorrhaging, deformities) [11,12] are consistent with lipid membrane and DNA damage. Symptoms of oxidative stress caused by HAH can also be alleviated by treatment with antioxidants [15].

Oxidative damage due to PAH metabolism may arise from the formation and subsequent redox cycling of quinones and from free radicals formed because oxygenation reactions are not 100% efficient [16]. It has been proposed that the toxicity of PAHs to larval fish is due to the prolonged induction of CYP1A enzymes associated with continuous exposure to PAHs and the eventual depletion of antioxidant defense systems [11].

Risk assessment models

Both alkyl-substituted and unsubstituted PAHs cause CYP1A induction [11,17,18], but the relative contribution of each to CYP1A induction or toxicity in a mixture is unknown. Thus, it is difficult to assess the risks associated with mixtures containing differing arrays and proportions of PAHs. Most current risk assessment models are simplified and do not consider multiple interactions. However, the concentration–addition model predicts additivity of responses in organisms ex-
posed to multiple components of a mixture by assuming that the components share a common mode of action [19]. For example, if 10 nM of chemical A and 50 nM of chemical B each caused a fivefold increase in EROD activity, a mixture containing 10 nM of chemical A and 50 nM of chemical B should cause a 10-fold increase if the components act additively.

The additivity theory has been incorporated into toxic equivalent factors (TEF) or induction equivalent factors, which have been used to assess the risks associated with mixtures of HAH, such as dioxins and furans [20,21]. The TEF approach is based on two assumptions underlying additivity. First, congeners within a class of chemicals act in an additive manner because they share similar mechanisms of toxicity; second, the Ah-receptor (AhR) is assumed to be the mediator of HAH-induced toxicity [22]. The assumption of a common mode of action is supported by exposure–response curves for chemically related compounds that are parallel and that have a similar range of response and efficacy. A further assumption is that TEFs for chronic toxicity to fish (blue sac disease) can be predicted from IEFs for CYP1A induction [13]. For PAH, correlations between EROD induction and early life stage toxicity are not yet established, so we have compared potencies of PAHs for EROD induction using IEFs.

In the TEF approach, chemical congeners are ranked according to their potency for causing a toxic effect in relation to the most toxic congener in that class. In the case of HAHs, 2,3,7,8-tetrachlorodibenzo-p-dioxin is considered the most potent congener, and its toxicological effects have been widely characterized [20]. The TEF of a specific HAH is expressed quantitatively as an equivalent concentration of TCDD needed to cause a similar effect. The TEF of a test compound is equal to EC50TCDD/EC50TEST, where EC50 is the median effective concentration of each chemical. The total toxic equivalent quantity (TEQ) of a mixture is the sum of the TEFs for each individual congener multiplied by their respective concentrations in the mixture:

\[ \text{TEQ}_{\text{mixture}} = (\text{TEF}_A \times \text{Concn}_A) + (\text{TEF}_B \times \text{Concn}_B) + \cdots + (\text{TEF}_N \times \text{Concn}_N) \]

[20]

The IEF is calculated the same way but uses IEFs in place of TEFs.

**Research outline**

We tested the null hypothesis that EROD activity in rainbow trout exposed to mixtures of alkyl-substituted and unsubstituted PAHs cannot be predicted by an additivity (concentration–addition) IEF model based on the induction potency of single compounds.

**MATERIALS AND METHODS**

**Approach**

Using benzo[k]fluoranthene (BkF) as a reference compound, we measured the IEFs for a group of alkyl-substituted and unsubstituted PAHs that persist in the environment and that induce a wide range of CYP1A activity in juvenile rainbow trout (Table 1). Subsequently, we measured CYP1A induction in trout exposed to mixtures of the same PAHs to determine if the IEF approach would be a suitable model for predicting mixture effects.

The potency of individual PAHs for causing CYP1A induction was assessed by 96-h bioassays of juvenile rainbow trout exposed to a range of PAH concentrations in water. Induction of CYP1A proteins was quantified via the EROD assay following brief exposures to waterborne PAHs [23]. A 96-h exposure accounts for differences in the kinetics and metabolism of the test compounds, based on the observation that EROD activity of retene-exposed trout reached a plateau maximum within 24 h [24]. Preliminary bioassays covering a broad range of concentrations (10-fold dilutions) were repeated iteratively, narrowing the exposure range and choosing exposure concentrations between those causing no induction and maximal induction, until the shape of the exposure–response curve was defined. As a consequence, some exposure concentrations were used once, while others (near the center of the curve) were repeated several times. The EC50 for each PAH was calculated from a regression relating log EROD activity to log concentration, calculated statistically using SigmaPlot® [25]. The number of fish per data point ranged from five to 20, depending on how frequently a given concentration was repeated in iterative bioassays. The IEFs were derived using BkF, the most potent compound tested, as a reference (IEF BkF = 1.0; Table 2). By expressing EC50s as IEFs, mixtures were prepared containing a fixed range of IEFs, and the potency of each mixture was tested using a bioassay. The suitability of the IEF model was assessed by how closely the measured EROD activity for mixtures matched the activity of BkF at the same number of total IEFs.

**Design of IEF model**

Based on their potency for EROD induction, PAHs selected for study were classified as strong (45- to 65-fold maximum induction, parallel exposure–response curves) or weak (three- to 37-fold maximum induction, various exposure–response

Table 1. Structures and physicochemical properties of polycyclic aromatic hydrocarbons (PAHs) examined

<table>
<thead>
<tr>
<th>Class of inducer</th>
<th>PAH ID</th>
<th>Log $K_{ow}$ [37, 38]</th>
<th>Molecular weight (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong inducers</td>
<td>Benzo[k]fluoranthene</td>
<td>BkF</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>Benzo[b]fluoranthene</td>
<td>Benze</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>2,3-Benz[a]fluorene</td>
<td>BbF</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>7-Isopropyl-1-methylphenanthrene</td>
<td>Retene</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>$\beta$-Naphthoflavone</td>
<td>BNF</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>Dibenzo[a,h]anthracene</td>
<td>DBA</td>
<td>6.8</td>
</tr>
<tr>
<td>Weak inducers</td>
<td>2-Ethyl-phenanthrene</td>
<td>2-ethyl</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>9-Ethyl-phenanthrene</td>
<td>9-ethyl</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>9-Ethyl-10-methyl-phenanthrene</td>
<td>9-ethyl-10-methyl</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>2-Methyl-phenanthrene</td>
<td>2-methyl</td>
<td>4.9</td>
</tr>
</tbody>
</table>
posed to three different concentrations of B
EROD activity were equivalent and their IEQs summed to 3.2 nM, that is, to 0.32, 1.0, and 3.2 IEQs. For a binary mixture, avone were obtained from Acros Organics (Fair Lawn, NJ, k
Benzo[a,h]two should cause 10-fold induction, while mixtures of four compounds. If these PAHs interacted additively, mixtures of strong inducers only, and mixtures combining weak and strong inducers. Each PAH was added to mixtures of strong inducers, and mixtures combining weak and strong inducers were carried in high-performance liquid chromatography-grade acetone.

**Table 2. Median effective concentrations (EC50s) and toxic equivalent factors (IEFs) for strong inducers**

<table>
<thead>
<tr>
<th>PAH*</th>
<th>EC50 (nM)</th>
<th>IEF (EC50_nom/-EC50_ren)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzo[k]fluoranthene (BkF)</td>
<td>7.2</td>
<td>1</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene (bzenec)</td>
<td>44.2</td>
<td>0.163</td>
</tr>
<tr>
<td>2,3-Benzo[b]fluorene (BbF)</td>
<td>98.7</td>
<td>0.073</td>
</tr>
<tr>
<td>β-Naphthoflavone (BNF)</td>
<td>132.2</td>
<td>0.054</td>
</tr>
<tr>
<td>Dibenz[a,h]anthracene (DBA)</td>
<td>190.7</td>
<td>0.038</td>
</tr>
<tr>
<td>7-Isopropyl-1-methylphenanthrene (retene)</td>
<td>207.4</td>
<td>0.035</td>
</tr>
</tbody>
</table>

*PAH = polycyclic aromatic hydrocarbon.

slopes) inducers. The IEF approach was used only to study mixtures of strong inducers because the exposure–response curves for the weak inducers were not parallel and showed lower maximal activity (see Results). The IEFs were derived as the ratios of the EC50 for BkF to the EC50s for each PAH, based on in vivo bioassays (Table 2). All mixtures were prepared in equipotent BkF-equivalent nominal concentrations of 0.32, 1.0, and 3.2 nM. Thus, the IEQ of PAH in any given mixture was either 0.32-, 1.0-, or 3.2-nM BkF equivalents. For example, in the BkF single-compound study, trout were exposed to three different concentrations of BkF: 0.32, 1.0, and 3.2 nM, that is, to 0.32, 1.0, and 3.2 IEQs. For a binary mixture, two PAHs were added in solution such that their potencies for EROD activity were equivalent and their IEQs summed to 0.32, 1.0, and 3.2 nM BkF (each component contributed one-half of the expected potency). The single PAH-exposed group was used as the baseline, and mixtures of PAHs consisted of two, four, and six compounds; for the six strong inducers, a total of 21 different mixtures were tested.

To assess the mixture effects of weak inducers, a similar experiment was conducted, but the measure of potency for each PAH was the concentration required to cause a fivefold induction of EROD activity. Three types of binary and quaternary mixtures were studied: mixtures of weak inducers only, mixtures of strong inducers only, and mixtures combining weak and strong inducers. Each PAH was added to mixtures at concentrations that should induce EROD activity by fivefold, as interpolated from exposure–response curves of single compounds. If these PAHs interacted additively, mixtures of two should cause 10-fold induction, while mixtures of four should cause 20-fold induction.

**Chemicals**

Retene (98% purity) was obtained from ICN Biomedicals (Costa Mesa, CA, USA); BkF (99% purity) and β-naphthoflavone were obtained from Acros Organics (Fair Lawn, NJ, USA); 2,3-benzo[b]fluorene (95% purity), benzo[b]fluoranthene (99% purity), and dibenz[a,h]anthracene (97% purity) were obtained from Sigma Aldrich (St. Louis, MO, USA); 2-ethylphenanthrene, 9-ethyl-10-methyl-phenanthrene, 9-ethyl-phenanthrene, and 9-ethyl-phenanthrene (purity unknown) were obtained from Sigma Aldrich rare chemicals. All PAH solutions were carried in high-performance liquid chromatography-grade acetone.

**Fish**

Juvenile rainbow trout (1–3 g) were selected as the test organism. Rainbow trout (Oncorhynchus mykiss) are prevalent in many inland water systems in Canada and are commonly used in toxicological studies. They are responsive to CYP1A induction by dioxins and furans [26] as well as by nonchlorinated PAHs [17]. They were purchased from Rainbow Springs Hatchery (Thamesford, ON, Canada) and acclimated for two to three weeks at 15°C in continuously flowing dechlorinated city water (pH = 7.88, hardness = 135 mg/L as CaCO3, salinity = 0.2 ppt, specific conductance = 321 μS/cm2, DO = 10.1 mg/L) at a flow rate of about 2 L of water per gram of fish per day. The light-dark photoperiod was 16:8 h, and the exposure chambers were covered to provide shade. Fish were fed a commercial trout diet (Martin’s Feed Mills, Elmira, ON, Canada) at a maintenance level of about 1.5% body weight per day. Fish were not fed 48 h before bioassays to avoid oxygen depletion or chemical absorption due to the presence of feces.

**Bioassays**

All bioassays were conducted according to methods described by Hodson et al. [23]. Five trout were placed in buckets containing a 10-L solution of PAH in dechlorinated water, and solutions were not aerated to avoid aerosols of PAH. The PAH solution and delivery water were renewed daily for 96 h. All buckets were housed in a controlled environmental chamber (15°C; 16:8-h light:dark photoperiod), and tanks were covered. Carrier controls consisted of fish exposed to 1 ml of acetone in 10 L of water.

After 96 h, fish were killed and weighed, and their livers removed and weighed immediately. Livers were homogenized in 500 μl of HEPES-KCl buffer (pH 7.4, 0.15 M KCl, 0.02 M HEPES), and the homogenate was centrifuged at 9,000 g for 20 min at 4°C in 1.5-ml microcentrifuge tubes. The resulting supernatant (S-9) fractions were removed, placed in cryovials, and stored in liquid nitrogen for EROD analysis.

**EROD assay**

The EROD activity was measured fluorimetrically by the deethylation of 7-ethoxyresorufin (7-ER) to resorufin, catalyzed by the CYP1A proteins in S-9 liver fractions [23]. The samples were assayed in triplicate in 96-well microplates. To each well, 50 μl of S-9 fraction and 50 μl of HEPES reaction buffer (10 μM 7-ER in 0.01 M HEPES buffer) were added. The plates were incubated for 10 min, and the reaction was initiated by the addition of 10 μl of 24 mM aqueous nicotinamide adenine dinucleotide phosphate. Fluorescence was measured once per minute for 12 min with a Cytofluor II® (Applied Biosystems, Foster City, CA, USA) microplate fluorometer (λexcitation = 530nm; λemission = 590nm). Each plate contained resorufin standards in duplicate. Positive controls included S-9 fractions from individual trout (5–10 g) injected with 10 μg β-naphthoflavone (BNF; a model inducer) per gram body weight prepared in sunflower oil; negative controls were S-9 fractions from trout injected with sunflower oil only.

Crude activity was calculated by measuring the slope of curves relating fluorescence to time, converting fluorescence to concentration by a resorufin standard curve, and expressing activity as pmol resorufin per minute. Molar specific activity (pmol resorufin/mg protein/min) was calculated by normalizing crude activity to S-9 protein concentrations, determined with Biorad Reagent (Biorad, Hercules, CA, USA) [23].

**Water fluorescence**

The PAH concentrations in water were measured with a QMI fluorescence spectrometer and FELIX© software (Photon
EROD induction by mixtures of PAHs

Technologies International, London, ON, Canada). A time-course study was conducted to characterize exposure concentrations after renewing solutions every 24 h. Exposure tanks, with or without fish, were dosed with a concentration of a PAH equal to its EC50, or the concentration causing EROD induction five times greater than controls. Water samples of 2.0 ml were placed in 7-ml glass vials at 0, 2, 4, 8, and 24 h post-PAH administration and diluted with high-performance liquid chromatography-grade ethanol (1:1, v/v). Vials were stored at −20°C in the dark to avoid photodegradation of PAH. Each sample was assayed for respective PAH concentrations using synchronous scan fluorescence [27]. The absolute concentration of each PAH was quantified by integrating relative peak areas, and changes in waterborne concentrations over time were expressed as a percentage of the initial concentration at time zero.

Statistical analysis

A p value < 0.05 was considered significant for all statistical tests. All data were log transformed for statistical analysis since the variance of EROD activity among treatments is non-homogeneous [23], as shown by the Bartlett test. The EC50s for each PAH were calculated using SigmaPlot [25], and all other statistical analyses were performed on log values using SigmaStat [28]. In the IEF study, a two-way analysis of variance was conducted to determine if there was a difference among equivalents and/or mixture type with respect to EROD activity. In the concentration–addition tests, one-way analyses of variance were conducted among all treatments exposed to a particular type of mixture (e.g., binary). For all analyses of variance, pairwise combinations were tested for significant differences using the Student–Newman–Keuls method. For each treatment, the 95% confidence limits about the geometric mean were calculated. Results were back-transformed to the original units (as geometric means with 95% confidence intervals). All results were reported as induction, that is, as the ratio of EROD activity in PAH-exposed fish to EROD activity of control fish, and induction was compared to the nominal concentrations of each PAH to derive EC50s.

RESULTS

With one exception, all PAHs added to bioassay tanks disappeared at comparable, exponential rates, and the amount of PAH in solution was less than 5% of nominal concentrations 24 h postaddition (Fig. 1). Concentrations of each PAH declined most rapidly in the presence of fish. For example, BkF was virtually absent from solution after 24 h when fish were present in the tank, but 50% of the added concentration remained when no fish were present (Fig. 1); similar effects were observed for the other PAHs (data not shown). The exception to this pattern was dibenz[a,h]anthracene, which showed only a slow rate of decline in concentration over 24 h (Fig. 1).

Exposure to individual PAH caused concentration-dependent increases in EROD activity (Figs. 2 and 3), relative to acetone carrier controls, which averaged 0.65 pmol/mg/min. Except for dibenz[a,h]anthracene, exposure–response curves of strong inducers (Table 1) were roughly parallel and showed similar values for maximal induction (Fig. 2). Using BkF as the reference compound, IEFs for these PAHs ranged from 0.03 to 0.16 (Table 2). For the four additional compounds (weak inducers; Table 1), exposure–response curves showed much lower slopes, and maximal activity was limited by mor-
tality at the highest exposure concentrations (Fig. 3). These chemicals were tested several times to ensure the results were not in error. In particular, for 9-ethylphenanthrene, the unusually narrow peak of activity was replicated several times.

When PAH exposure was expressed as IEQs, exposure–response curves for all six strong inducers appeared similar, but with some variability (Fig. 4). Fish also showed the same response when exposed to binary mixtures. For example, EROD activity of fish exposed to 0.32 to 3.2 nM of B\(k\)F was the same as that of fish exposed to 0.32 to 3.2 nM IEQ of benzo[\(b\)]fluorone or to a 0.32–3.2-nM equipotent mixture of B\(k\)F and benzo[\(b\)]fluorone (Fig. 5).

When several PAHs were mixed at concentrations giving 0.32- to 3.2-nM IEQs, exposure–response curves of mixtures appeared quite similar to exposure–response curves for single PAH (Fig. 6). This pattern held true for six single compounds, six mixtures of two compounds, four mixtures of four compounds, and one mixture of six compounds (total of 17 tests; Fig. 6). For each mixture, the sum of the concentrations were equal to 0.32, 1.0, or 3.2 IEQs.

When only strong or only weak inducers were mixed to-}

DISCUSSION

Exposure to PAHs (single and mixtures) caused significant CYP1A induction in trout, as measured by EROD activity (Figs. 2 and 3). No significant levels of enzyme induction were

![Graph of Fig. 4](image1)

![Graph of Fig. 5](image2)

![Graph of Fig. 6](image3)

![Graph of Fig. 7](image4)
associated with the noninduced (control) fish. The EROD activity of internal and external controls and induction by single PAH was consistent with values obtained in an experiment comparing induction in vivo to induction in vitro [17]. Although exposure concentrations in each bioassay were not constant after daily renewal of solutions, exposure regimes were consistent among chemicals, except for dibenz[a,h]anthracene. Further, responses of fish appeared highly repeatable for either strong or weak inducers and for single compounds or mixtures. Despite differences among log $K_{ow}$ values (Table 1), the relative rate of loss of PAH from initial concentrations was similar for all compounds, and most of the decline was attributed to uptake by fish, as shown by the example of BkF, which disappeared more rapidly in the presence than in the absence of fish (Fig. 1). Hence, exposure could be characterized either as the initial concentration of PAH or as the area under the concentration–time curves; for simplicity, we have characterized exposure as the initial or nominal concentration.

These results did not support the null hypothesis, that is, that CYP1A induction by PAH mixtures could not be predicted using an IEF model. When the conditions of the model were met, that is, when PAH mixtures were composed of compounds of similar slope and efficacy (Figs. 5 and 6), mixtures of PAHs demonstrated simple additivity, predictable from IEFs. However, when mixtures of PAHs were composed of compounds with differing slopes and efficacy, EROD activity of exposed fish exceeded the predicted value. Thus, the null hypothesis was accepted for these mixtures. To assess the induction associated with mixtures composed of unlike PAHs, it may be more accurate to conduct bioassays rather than to predict their potency using a model.

**IEF study**

The IEFs derived from studies of single compounds were an effective means of expressing relative potency because there were no significant differences in potency for CYP1A induction among individual PAHs at a given IEQ. This trend was also evident in the mixture exposures; in most cases, there were no significant differences in CYP1A activity among fish exposed to all two-, four-, or six-component mixtures at a specific IEQ. Therefore, bioassays of fish exposed to single compounds could be used to predict CYP1A induction in mixtures of PAHs that meet the IEF criteria.

One exception was noted among strong inducers: The exposure–response slope of dibenz[a,h]anthracene was different from that of other strong inducers. The effects of this difference were noted in mixtures that contained dibenz[a,h]anthracene; often the induction associated with these mixtures was statistically different from expected values (data not shown). This reinforces the assumption that the IEF model applies only to like compounds. The source of this difference may be the unexpected persistence of dibenz[a,h]anthracene in water. Unlike the other PAHs, its concentration did not decline rapidly after addition to water, despite a relatively high $K_{ow}$ (Table 1), and this behavior would create a different dose–time–response relationship.

Weak inducers could not be included in the IEF approach because they had varying exposure–response slopes and their maximum activities were not as consistent nor as high as those of strong inducers. Nevertheless, within this group, there appeared to be additivity in CYP1A induction. However, with mixtures containing both strong and weak inducers, CYP1A activity was induced to levels significantly greater than expected. This raises the question of whether the IEF model can be applied accurately to assess aquatic environments contaminated with PAHs of a wide range of potency and physico-chemical characteristics.

**Interpretations of findings**

The assumption of an IEF approach is that there is additivity among mixture constituents due to a common mechanism of action, parallel exposure–response curves, and a similar range of CYP1A induction by components of the mixture [20,21]. A qualitative examination of the concentration–response curves by single strong inducers (Fig. 2) showed parallel response curves (except for dibenz[a,h]anthracene) and a similar range of EROD induction. Therefore, the strong inducing PAH met the basic criteria necessary for an IEF approach, and the model accurately assessed the induction caused by their mixtures. However, PAHs of varying potency exist in nature, and this approach may not be applicable to the entire family of PAH compounds.

Greater-than-additive EROD activity in the mixtures of strong and weak inducers may be explained by the following. First, PAHs do not conform to the additive model of response that has been proposed for chemicals that act via a common receptor. It has been generally accepted that carcinogenicity of many PAHs is mediated via the AhR [29] and the production of CYP1A proteins. Our application of IEF to PAH is based on a model in which chronic toxicity to larval fish is linked to the interaction of PAH with the AhR, and toxicity follows DNA signal transduction due to oxidative stress arising from increased CYP1A activity and accelerated oxygenation of PAH. However, if alternate pathways or receptors exist, additivity of toxicity in PAH mixtures may not occur. Verhaar et al. [30] and Matsumara [31] stated that alternate pathways, such as narcosis and protein phosphorylation, may mediate the toxicity of PAH compounds. In studies with rats, specific responses (hyperthyroidism and wasting syndrome) have been noted that are difficult to correlate with CYP1A induction. Further investigations are needed to identify how PAHs exert their toxic effects on organisms and whether CYP1A induction is an accurate predictor of toxicity.

Second, other studies have shown that synergistic reactions within aromatic hydrocarbon mixtures can occur. Hermann [32] determined that numerous PAHs (such as chrysene, benzo[b]fluoranthene, and benzo[a]pyrene) could increase the mutagenicity of benzo[a]pyrene, benzo[a]anthracene, and 3-methylcholanthrene to greater-than-additive levels. Till et al. [33] illustrated that EROD activity of a mixture of 16 PAHs in rat hepatocyte cultures was about two times greater than expected based on IEFs and proposed complex interactions among PAH chemicals and unidentified contaminants. Compounds such as PAH, dioxins, and steroid hormones can also up- or down-regulate receptor levels to modulate receptor-mediated effects [34,35]. Bannister and Safe [36] found that when a binary mixture of TCDD and 2,2′,4,4′,5,5′-hexachlorobiphenyl was administered to mice, a significant increase occurred in EROD activity due to increased AhR levels within 2 d of treatment. If some PAHs also increase the number of AhRs, more receptor sites would be available for binding by mixture components, resulting in a greater-than-expected EROD induction.

A third reason why nonadditive induction was observed may be the complex chemical and physical interactions among PAHs in a mixture. Differences in toxicokinetics, toxicodyn-
amics, and metabolic effects among the chemicals within a mixture will affect the overall toxicity, and these are not accounted for in the IEF model. For example, chemicals in a mixture will have different rates of uptake, metabolism, time to reach steady state, and affinities for the AhR (J.C. Orr, Can Tox, unpublished data). In particular, given the slow rate at which dibenz[a,h]anthracene disappeared from solution, pharmacokinetics could explain why it did not conform to the IEF model. These differences and previous studies of induction in fish cells [18; N.C. Bols, University of Waterloo, personal communication] support our findings that PAH mixtures may act in a nonadditive manner.

**Significance of study**

In the environment, alkyl-substituted and unsubstituted PAHs of varying potency and efficacy occur as complex mixtures, and there is little research on their biological effects and interactions. Thus, methods are needed to rapidly assess the ecological risks of PAH mixtures. We have used the IEF approach to determine if the potency of mixtures of PAHs for CYP1A induction can be predicted from the relative potency of each component. CYP1A induction by mixtures of PAHs that have similar slopes and efficacy was predicted successfully by the IEF model, but not induction caused by mixtures of PAHs having differing slopes and efficacy. Thus, to assess the risks of mixtures that contain PAHs with different properties, bioassays of the entire mixtures appear to be a more valid, direct, and accurate approach than an IEF model. To substantiate this conclusion, further research is needed to completely characterize the toxicokinetics of PAHs and their interactions with the AhR receptor of fish, individually and in mixtures.

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