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Characterization of the H4IIE Rat Hepatoma Cell Bioassay for Evaluation of Environmental Samples Containing Polynuclear Aromatic Hydrocarbons (PAHs)

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Abstract. The H4IIE rat hepatoma cell bioassay has been extensively used to assess the toxic equivalents (TEQs) of complex mixtures of halogenated aromatic hydrocarbons in environmental samples. However, there is often a discrepancy between bioassay induction results and toxic equivalents calculated from chemical analysis of samples; the former generally yield higher bioassay-TEQs. Polynuclear aromatic hydrocarbons (PAHs) are a class of chemicals which can significantly contribute to induction-TEQs. Benzo(a)pyrene (BAP), dibenz(a,h)anthracene (DBA), benz(a)anthracene (BA), benzo(k)fluoranthene (BkF), benzo(b)fluoranthene (BbF), chrysene (Chr), and indeno(1,2,3-c,d) pyrene (IdP) are carcinogenic PAHs found in environmental samples, including oysters collected from Galveston Bay. The induction potency of these PAHs relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was determined individually in rat hepatoma H4IIE cells seeded in 6-well plates, and the induction-derived equivalency factors (EFs) relative to TCDD were 0.000354, 0.00203, 0.000025, 0.00478, 0.00253, 0.00020, 0.0011 for BAP, DBA, BA, BkF, BbF, Chr, and IdP, respectively. Dilutions of a reconstituted PAH mixture containing 23 PAHs (744 to 4466 ng/g total PAHs) with constant percentages of BAP (4.5%), DBA (3.5%), BA (2.4%), BkF (3.7%), BbF (3.5%), Chr (4.7%), and IdP (4.2%) yielded bioassay-derived induction-EQs that ranged from 0.52 to 1.44 ng/g. Oysters exposed in the laboratory to the same PAH mixture for 30 days differentially accumulated the PAHs with time. Bioassay-EQs for these oyster extracts ranged from 0.94 to 5.79 ng/g. These results were similar to the chemically calculated EQs which varied from 0.81 to 3.13 ng/g.

The H4IIE rat hepatoma cell bioassay has been used to characterize the induction potency of various halogenated aromatic hydrocarbon (HAH) mixtures containing polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), and dibenzofurans (PCDFs) (Zacharewski *et al*. 1989; Tysklind *et al*. 1994; Tillitt *et al*. 1991; Schmitz *et al.* 1995). HAHs bind to the aryl hydrocarbon (Ah) receptor which in turn initiates CYP1A1 gene transcription upon binding to the CYP1A1 regulatory region on DNA (Whitlock 1990). Induction of CYP1A1 can be quantitated by fluorimetric measurement of ethoxyresorufin-*O*-deethylase (EROD) activity. H4IIE cells have low basal CYP1A1-dependent activity but are highly inducible by HAHs allowing for very sensitive assays. For example, the limit of detection for induction of EROD activity by 2,3,7,8-tetrachloro-p-dibenzodioxin (TCDD) was 31 fmol using 15 × 100 mm petri dishes (Tillitt *et al*. 1991).

Because the various HAH congeners bind to the Ah receptor and elicit a number of common toxic and biochemical responses, a toxic equivalency (TEQ) approach has been developed to describe the potency of complex mixtures of HAHs. 2,3,7,8-TCDD is the most potent inducer of EROD activity in H4IIE cells and is used as a reference standard for developing toxic equivalency factors (TEFs) for the induction activity of individual HAHs (Safe *et al*. 1989; Ahlborg *et al*. 1994). The TEF approach can be used also to calculate TEQs for HAH mixtures (*i.e*., $TEQ_{extract} = \{[HAH]_1TEF_1 + \ldots + [HAH]_nTEF_n\}$) and several studies have shown that there is a good correlation between calculated and observed TEQs for HAH mixtures (Tillitt *et al*. 1991; Safe *et al*. 1989). In contrast, it has also been reported that chemically calculated TEQs for some mixtures were lower than bioassay-derived TEQs for the same samples (Zacharewski *et al*. 1989; Kennedy *et al*. 1996; Schmitz *et al*. 1996; Williams *et al*. 1995) which may be indicative of either synergistic interactions of the contaminants and/or the presence of active compounds which are not identified by chemical analysis. PAHs are a class of compounds identified in some environmental samples, and these compounds induce EROD activity in both *in vivo* and *in vitro* bioassays (Van der Weiden *et al*. 1994; Piskorska-Pliszczynska *et al*. 1986; Brunstrom *et al*. 1991; Machala *et al*. 1996). The current study reports induction EFs for seven (Figure 1) of the most active PAHs. The concordance between chemically calculated and bioassay induction EQs was tested by exposing oysters collected from Galveston Bay to a reconstituted mixture of PAHs.

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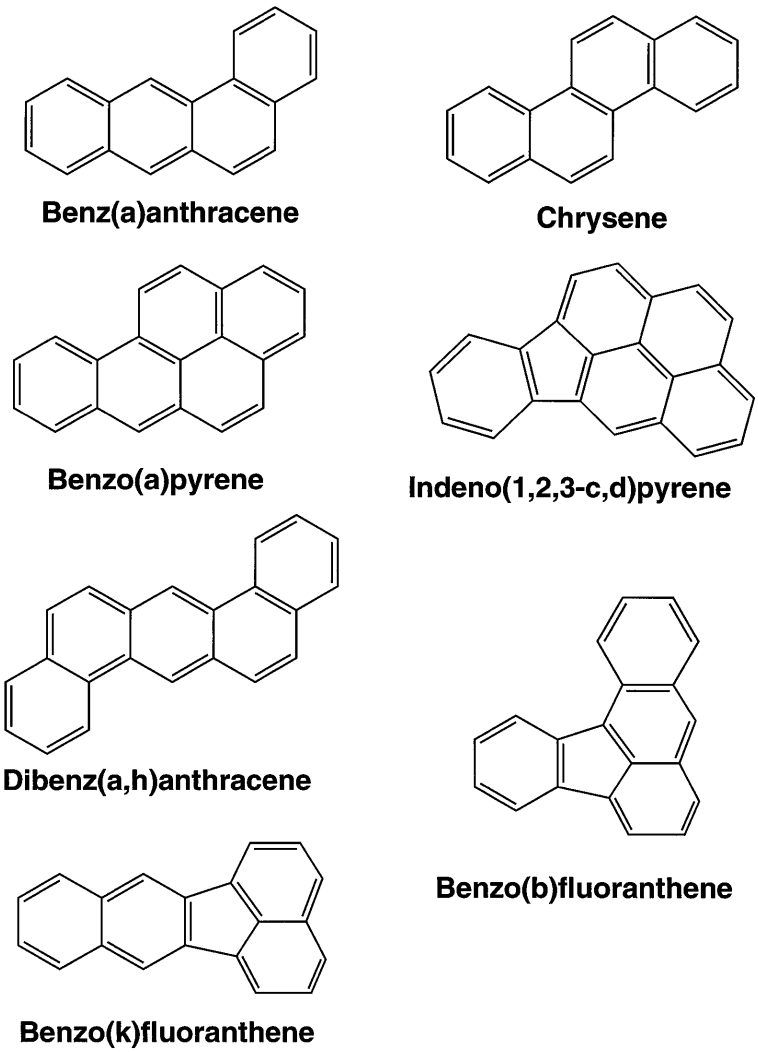

Fig. 1. Structures of selected polynuclear aromatic hydrocarbons

Materials and Methods

Cell Culture and EROD Assay 6-well Plate Protocol

Rat hepatoma H4IIE cells were grown as a continuous cell line in α-minimum essential media (Sigma, St. Louis, MO), supplemented with 2.2-g/L sodium bicarbonate, 10% fetal bovine serum, and 10-ml/L antibiotic/antimycotic solution. Stock culture cells were grown in 150-cm^2 plates at 37°C in a humidified air/carbon dioxide (95/5%) atmosphere. The cells were seeded in 6-well plates in 2-ml media per well. After 24 h, the cells were treated with two 5-µl test extracts, PAH standards, or 1-nM 2,3,7,8-TCDD dissolved in DMSO. 2,3,7,8-TCDD was used as the reference standard for each experiment. After incubation for 24 h, the media was removed and the cells were washed with 2-ml Hanks (Sigma, St. Louis, MO) solution and harvested by scraping into 4-ml Tris-sucrose buffer (Tris base 6.05 g/L, sucrose 59.5 g/L, pH 8.0). The cellular pellet was isolated after centrifugation for 5 min at 1000 rpm at 4°C and resuspended in 200-µl buffer. Fifty-microliter aliquots of the cell suspension were used for both EROD assays and protein determinations. EROD assays were determined as described by Pohl and Fouts (1980) with modification. The 1.15-ml incubation mixtures in 0.1 M HEPES, pH 8.0, contained 0.1-mg NADPH, 0.1-mg NADH, 1.5-mg BSA, 0.7-mg $MgSO_4$, and 50 µl of cells. Fluorescent metabolites were determined using a spectrofluorometer at 550-nm excitation and 585-nm emission wavelength settings. Protein concentrations were determined by the method of Bradford (1976).

Calculation of Induction EFs and EQs

EC_{50}s were calculated by both probit analysis and from a Gaussian curve fit of the induction data. The Gaussian distribution was a better description of the data, so those results are reported in this study. The Gaussian curve fitting procedure was similar to the one described by Kennedy and coworkers (1993), and Sigma Plot (Jandel Scientific) was used to perform the calculations. The equation is as follows:

$$y(d) = Y_b + (Y_m - Y_b)\exp\{-C[\ln(\mathrm{d}) - \ln(d_m)^2\}$$

where

$$C = \frac{\ln(2)}{(\ln(\mathrm{EC}_{50}) - \ln(d_m))^2}, \text{ and}$$

$y(d)$ is EROD activity at the PAH concentration d, Y_b and Y_m are basal and maximal EROD activity, respectively, and d_m is the PAH concentration when the EROD activity is maximal. Because BA and Chr did not cause depressed EROD activity at the highest concentrations used, a

logistic function was used to fit the EROD dose response curves for these compounds:

$$y(d) = Y_b + (Y_m - Y_b)\{1 + \exp[-(\ln(d) - \ln(EC_{50}))]^2\}^{-1}$$

where the symbols are as previously described. The induction-EF for each individual PAH was calculated by dividing the EC_{50} for TCDD by the EC_{50} for each PAH. The chemically derived induction EQ was calculated as described below:

$$EQ_{extract} = \Sigma([PAH]_1EF_1 + \cdots + [PAH]_nEF_n)$$

Aquarium Experiment

The aquarium exposure experiment conditions and PAH concentration data used for this investigation were previously reported elsewhere (Sericano 1993). Briefly, American oysters (*Crassostrea virginica*) were collected by dredge from Hanna's Reef, Galveston Bay, Texas, transferred as soon as possible to 40-L glass aquariums, and adapted to laboratory conditions for 7 days prior to the experiments. A total of eighty oysters were divided in two separate tanks. The oysters in the PAH-dosed tank were exposed to a mixture of 23 PAHs (prepared by dilution of NIST SRM 1491, National Institute of Standards and Technology, Gaithersburg, MD, USA) adsorbed onto Kaolin particles. The relative percent composition of the dosing mixture is shown in Table 1. The uptake experiment was performed at one dosing level and the nominal concentrations of suspended solids and total PAH in the dosing mixture were 10 mg l^{-1} and 240 µg g^{-1}, respectively. The nominal aquarium total PAH concentration was 2.4 µg l^{-1}. The oysters were continuously fed during the course of the experiment with a mixture of two algae, *Thalassiorsia fluviatilis* and *Isochrysis galbana*. Temperature, pH, salinity, suspended particles, and recirculation flow for each aquarium were monitored daily. The control tank was prepared identically to the test tank, but no PAHs were added. The exposure study lasted one month, during which groups of five oysters were collected from each aquarium during the 3rd, 7th, 15th, and 30th day. The reported concentrations correspond to a single determination of five pooled bivalves.

Chemical Extraction and Analysis

The analytical procedure for the extraction and fractionation of the PAHs in the oyster tissues was based on a previously reported method (Wade *et al*. 1993). Each batch of samples (8–10) was accompanied by the appropriate QA/QC samples including a minimum of a procedural blank, laboratory spiked blank, and/or reference material that were carried throughout the entire analytical procedure. Before extraction, PAH internal standards (d_8-naphthalene, d_{10}-phenanthrene, d_{10}-acenaphthene, d_{12}-chrysene, and d_{12}-perylene) were added to all samples, blanks, and spiked blanks or reference samples. Approximately 15 g of wet tissue were used for the analysis. After the addition of 50 g of anhydrous sodium sulfate, the tissue was extracted sequentially with three 100-ml portions of methylene chloride using a homogenizer. The extracts were concentrated to 2-ml hexane and separated into two fractions using alumina/silica gel chromatography. The first fraction containing aliphatic hydrocarbons was eluted using 45 ml of pentane and discarded. The PAH-containing fraction was then collected by eluting the column with 200 ml of a 1:1 mixture of pentane:methylene chloride. Further purification of the tissue extracts was performed by size exclusion chromatography on a Sephadex LH-20 column (Ramos and Prohaska 1981). PAHs were quantitatively determined by GC/MSD in selected ion monitoring (SIM) mode using the molecular ions and at least one confirmation ion for the components of interest (Wade *et al*. 1993). A 30-m DB-5 capillary column (0.32 mm i.d., 0.052-m film thickness) was temperature programmed from 40 to 300°C at 10°C min^{-1} and held at 300°C for 10 min. The GC/MSD was calibrated by injections of standard solutions at four different concentrations. Analyte identity was confirmed by molecular weight and retention time of authentic standards. In this study, BkF and BbF were not completely resolved, so the concentration represented by the peak complex was equally divided before use in EQ calculations.

Table 1. Reconstituted PAH mixture used in oysters exposure studies

Compound	Percent Contribution
Naphthalene	4.63
1-Methylnaphthalene	5.58
Biphenyl	4.70
2,6-Dimethylnaphthalene	4.84
Acenaphthylene	4.68
Acenaphthene	4.89
2,3,5-Trimethylnaphthalene	4.43
Fluorene	4.88
Phenanthrene	4.71
Anthracene	5.25
1-Methylphenanthrene	4.70
Fluoranthene	3.97
Pyrene	3.96
Perylene	4.78
Benzo(g,h,i)perylene	3.55
Benzo(e)pyrene	3.78
Benz(a)anthracene	2.41
Chrysene	4.72
Benzo(a)pyrene	4.56
Indeno(1,2,3-c,d)pyrene	4.23
Dibenz(a,h)anthracene	3.48
Benzo(b)fluoranthene	3.53
Benzo(k)fluoranthene	3.74

Results

Curves for induction of EROD activity by TCDD and two representative PAHs are illustrated in Figure 2. The curve for TCDD had a Gaussian distribution and an EC_{50} of 1.1×10^{-10} *M*. BkF was the most potent PAH with the lowest EC_{50} while benz(a)anthracene (BA) was the weakest inducer of EROD activity of the seven PAHs tested (Table 2). A logistic fit was used for the BA and chrysene curves because the highest concentrations did not cause a decrease in EROD activity. All the PAH curves show maximal induction of EROD activities comparable to that observed for TCDD. However, the overall order of induction potency based on the EC_{50}s of the EROD induction curves was BkF > BbF > DBA > IdP > BaP > Chr > BA (Table 2). Due to their higher affinity for the Ah receptor than PAHs of lower molecular weight (Piskorska-Pliszczynska *et al*. 1986), it was hypothesized that these seven PAHs would account for the majority of the EROD induction activity in extracts containing complex mixtures of PAHs.

To test the ability of the bioassay to predict the chemically calculated EQs, dilutions of the reconstituted PAH mixture (Table 1) to produce concentrations that were within the range of concentrations found in oysters in the environment (*i.e*., 700 to 4500 ng/g total PAHs dry weight) (Sericano 1993) were analyzed in the H4IIE bioassay. Bioassay EQs ranged from 0.521 to 1.443 ng/g. The chemically-calculated EQs ranged from 0.306 to 1.837 ng/g dry weight. There was a good

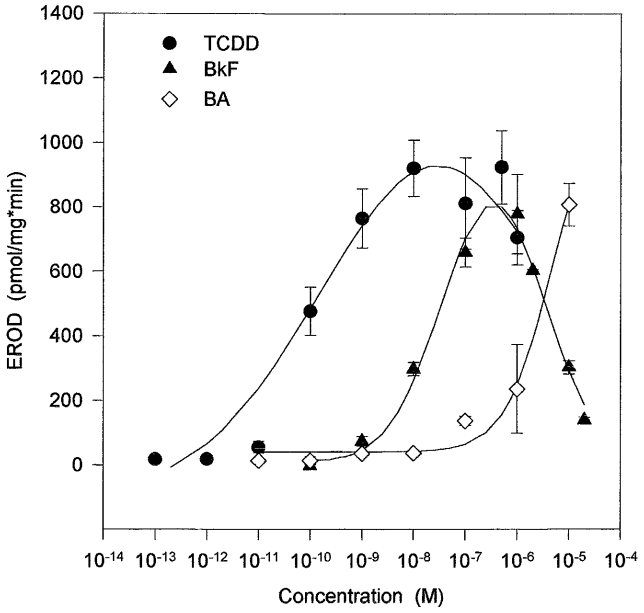

Fig. 2. Representative EROD induction curves for TCDD, benzo(*k*)fluoranthene (BkF), and benzanthracene (BA). EROD activities in the treated H4IIE rat hepatoma cells and the curve fitting procedure were as described in the Materials and Methods section

Table 2. Selected PAH Equivalency Factors

	EC_{50} (M)	Induction EF
BA	4×10^{-6} [a]	0.000025
Chr	5×10^{-7} [a]	0.00020
BaP	3×10^{-7}	0.000354
IdP	1×10^{-7}	0.00110
DBA	5×10^{-8}	0.00203
BbF	4×10^{-8}	0.00253
BkF	2×10^{-8}	0.00478
TCDD	1.1×10^{-10}	1

[a] Derived using a logistic function

correlation between the calculated and the bioassay induction EQs for this mixture (Figure 3). The seven PAHs used to calculate the chemical EQs constituted 27% by weight of the total mixture, while the chemical EQs from these seven PAHs accounted for 41 to 128% of the bioassay EQs for this mixture. The agreement among the EQs for the mixture indicates that these seven PAHs are responsible for most of the induction potency of the mixture.

The concentration of total PAHs in the exposed oysters ranged from 289 ng/g in the control day 0 to 2970 ng/g after exposure to the PAH mixture for 30 days (Table 3). The corresponding bioassay EQs and chemically calculated EQs ranged from 0.6 ng/g to 5.8 ng/g and 0.03 ng/g to 3.1 ng/g, respectively. Comparison of the bioassay and calculated EQs for extracts from PAH-exposed oysters is illustrated in Figure 4. The EQs derived using the control oysters were considered background and subtracted in order to obtain the net induction effects resulting from PAH exposure.

The seven PAHs constituted an increasing proportion of the total PAH concentration in the oysters over time (Tables 3 and 4), and this is reflected in the proportion of the bioassay EQs explained by the sum of the seven calculated EQs. When EQs are compared for PAH-exposed oysters, the ratio for bioassay to chemically calculated EQs ranged from 0.5 to 4.1 (Figure 4). However in the oysters exposed to the PAH mixture for 30 days, the seven PAHs in the EQ calculations constituted 58% of the total PAHs in the oyster extracts, and the bioassay EQ was only 1.7-fold higher than the chemically calculated EQ for PAHs in these oysters. The higher bioassay EQs indicate that there may be other active inducers present in the oyster extracts that have not been assigned EFs and/or are not detected by conventional chemical analysis.

Discussion

PAHs derived from both combustion and petroleum sources are widespread environmental contaminants. High concentrations of PAHs have been reported in sediments or extracts of marine invertebrates which have a low capacity for PAH metabolism and excretion (Livingstone 1985). PAH concentrations in oysters collected from Galveston Bay have been reported as high as 3,100 ng/g (Jackson *et al*. 1994) to 4,466 ng/g (Sericano 1993). PAHs can influence an induction bioassay if they are not removed from an environmental extract (typically by acid treatment). For example, an induction bioassay for HAHs in a mixture that also contains PAHs will overestimate the HAH contribution to the TEQ and, therefore, it is important to consider the presence of both classes of chemicals when analyzing environmental samples for risk assessment purposes. Structure activity studies of HAHs have indicated that there is a good correlation between structure-induction (EROD activity) and structure-toxicity relationships for these compounds (Safe *et al*. 1989). In this study the order of induction potency for the individual PAHs was BkF > BbF > DBA > IdP > BaP > Chr > BA and this was not correlated with their carcinogenic potency. Nisbet and LaGoy (1992) have proposed a set of genotoxic equivalency factors for the PAHs based on *in vivo* carcinogenicity data. DBA was the most carcinogenic compound, followed by BAP, while BA, BbF, BkF, and IdP had equivalent TEFs followed by Chr. The lack of correlation between genotoxicities and induction potencies is not surprising due to their different mechanisms of action.

BkF was the most potent inducer of EROD activity with an induction EF that is 0.005 relative to TCDD. Clean oysters at the beginning of the exposure study had only 2 ng/g-BkF or 0.6% of the total PAHs. After a 30-day exposure to the reconstituted PAH mixture containing 3.74% BkF, concentrations of this compound in oysters were 372 ng/g BkF or 13% of the total PAHs. The preferential bioconcentration of the higher molecular weight PAHs was observed for all seven of the PAHs reported in this study. While these seven PAHs (BkF, BbF, DBA, IdP, BaP, Chr, BA) comprised 27% of the PAH mixture, they made up 58% of the total PAHs in extracts from the exposed oysters. Bioaccumulation of high molecular weight PAHs has also been reported in oysters transplanted from clean to contaminated sites in Galveston Bay, Texas, and Dorchester and Duxbury Bays in Massachusetts (Sericano 1993; Peven *et al*. 1996).

In order to compare bioassay and chemically calculated TEQs, the reconstituted PAH mixture was diluted to 5 concentrations ranging from 744 ng/g to 4466 ng/g total PAHs. There was less than a twofold difference between the bioassay and

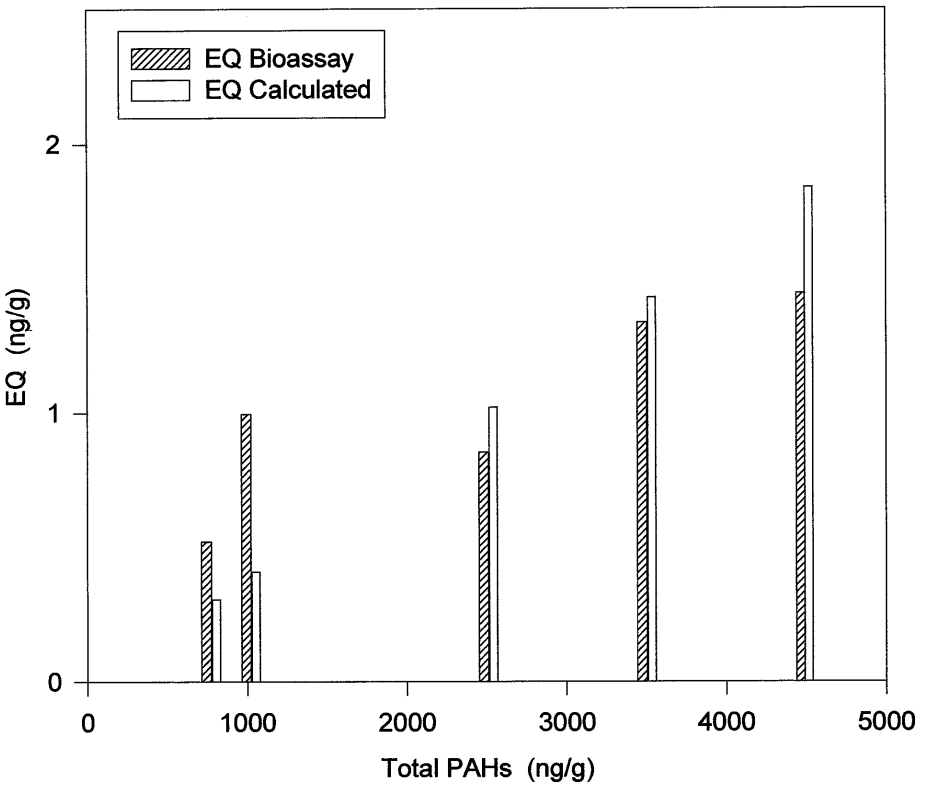

Fig. 3. Bioassay-derived and chemically calculated induction equivalents (ng/g dry weight) of the reconstituted PAH mixture in H4IIE rat hepatoma cell bioassay

Table 3. Uptake of PAH mixture by oysters over 30 days [a]

Compound	Day 0	Day 3	Day 7	Day 17	Day 30
	ng/g dry weight				
2-ring PAHs	96	274	229	157	107
3-ring PAHs	112	353	353	308	336
Fluoranthene	11	84	91	76	144
Pyrene	16	86	82	88	83
Perylene	3	9	12	22	47
Benzo(g,h,i)perylene	7	36	27	65	144
Benzo(e)pyrene	4	104	92	154	430
Benz(a)anthracene	8	85	67	154	305
Chrysene	8	52	55	112	286
Benzo(a)pyrene	6	29	16	35	97
Indeno(1,2,3-c,d)pyrene	10	73	47	102	200
Dibenz(a,h)anthracene	3	19	10	26	45
Benzo(b)fluoranthene	2	92	77	150	372
Benzo(k)fluoranthene	2	92	77	150	372
Total PAHs (ng/g)	289	1387	1234	1600	2967

[a] Data from Sericano (1993)

chemically calculated EQs for this PAH mixture over this range of concentrations (Figure 3). The good agreement between the two methods indicates that the seven PAHs for which EFs have been derived represent most of the induction potency of the reconstituted mixture. Using oyster extracts, bioassay and chemically calculated EQs differed by less than 4.1-fold. After exposure to the PAH mixture for 30 days, the oyster extracts contained higher concentrations of the more active PAHs than at the earlier time-points, and the difference in the bioassay and the calculated EQs was only 1.7-fold. There may be several reasons for the differences between calculated versus the bioassay EQs values. EFs for individual PAHs were determined from their EC_{50} values whereas a comparison of their potencies at lower doses (*i.e.*, EC_{10}) may be more relevant particularly when samples exhibit relatively weak induction activity. Moreover, it is impossible to identify all of the compounds in an environmental sample, and therefore an unidentified compound will increase bioassay EQs compared to calculated EQs. Interactions between inactive and active inducers or among active inducers of P450-dependent activities can also contribute to differences in EQs. The underlying premise for the EQ approach assumes strict additivity among inducers and this may not always be the case. However, the results obtained in this study using a defined PAH mixture (Figure 3), or extracts from PAH exposed oysters (Figure 4), show that the bioassay and calculated EQs differed by less than fivefold when only seven PAH induction EFs were used for the calculated EQs. This result also suggested that the seven AhR agonists additively accounted for most of the induction caused by PAHs in both the reconstituted mixture and oyster samples.

In conclusion, induction EFs have been reported for the first time in the H4IIE bioassay for a series of PAHs that typically occur in environmental samples and hence influence induction EQ estimates for these samples. Several PAHs were potent inducers of EROD activity and there was a good concordance between bioassay and chemically calculated EQs for both the reconstituted PAH mixture and extracts from oysters exposed to this mixture. Therefore, the H4IIE bioassay is generally additive in response to samples containing only PAHs. Some bioassays using HAHs have suggested more complicated interactions than simple additivity (Zacharewski *et al*. 1989; Kennedy *et al*. 1996; Schmitz *et al*. 1996; Williams *et al*. 1995). Future research will investigate how the bioassay and chemical

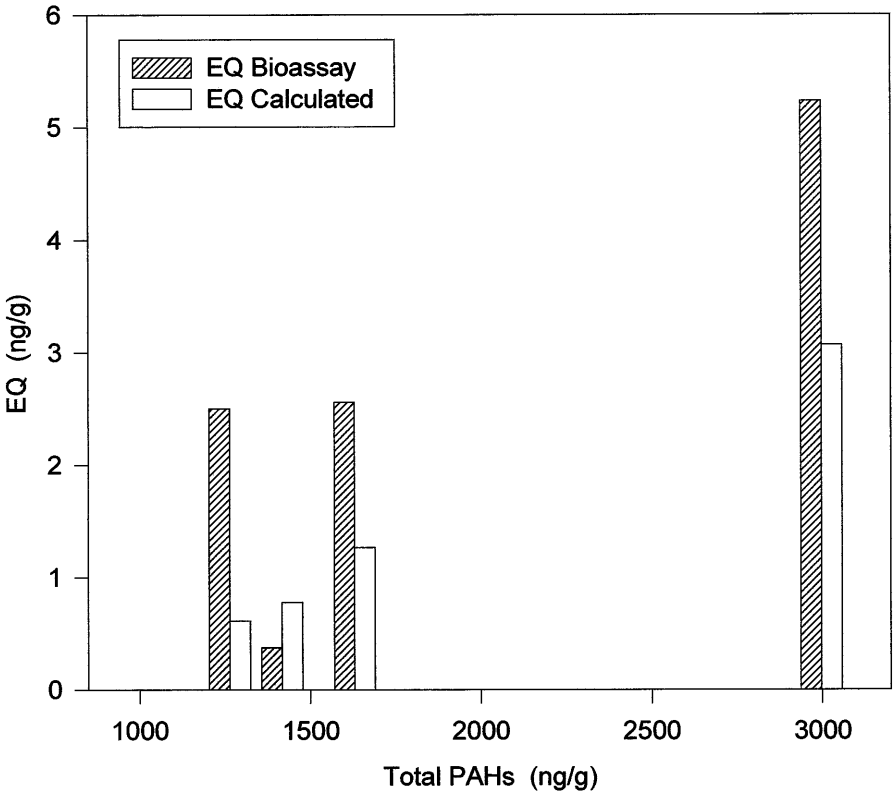

Fig. 4. Bioassay-derived and chemically calculated induction equivalents (ng/g dry weight) of the extracts of PAH-exposed aquarium oysters in H4IIE rat hepatoma cell bioassay

Table 4. Uptake of PAH mixture by oysters over 30 days[a]

	Day 0	Day 3	Day 7	Day 17	Day 30
Compound	Percent of Total PAHs				
2-ring PAHs	33	20	19	10	4
3-ring PAHs	39	25	29	19	11
Fluoranthene	4	6	7	5	5
Pyrene	6	6	7	6	3
Perylene	1	0.6	1	1	2
Benzo(g,h,i)perylene	2	3	2	4	5
Benzo(e)pyrene	1	8	8	10	14
Benz(a)anthracene	3	6	5	10	10
Chrysene	3	4	4	7	10
Benzo(a)pyrene	2	2	1	2	3
Indeno(1,2,3-c,d)pyrene	3	5	4	6	7
Dibenz(a,h)anthracene	1	1	0.8	2	2
Benzo(b)fluoranthene	0.6	7	6	9	13
Benzo(k)fluoranthene	0.6	7	6	9	13

[a] Data recalculated from Sericano (1993)

EQs correlate in oysters environmentally exposed to both HAHs and PAHs using a more sensitive and automated assay procedure in which EROD activity and protein determinations can be done simultaneously (Kennedy and Jones 1994) using a Cytofluor 2350 fluorescence plate reader.

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