TOXICOLOGICAL SCIENCES **88(2)**, 400–411 (2005)
doi:10.1093/toxsci/kfi338
Advance Access publication September 21, 2005

Formulation and Characterization of an Experimental PCB Mixture Designed to Mimic Human Exposure from Contaminated Fish

Paul J. Kostyniak,*,[1] Larry G. Hansen,† John J. Widholm,‡ Rich D. Fitzpatrick,* James R. Olson,* Jennifer L. Helferich,* Kyung Ho Kim,§ Helen J. K. Sable,† Rich F. Seegal,[¶] Isaac N. Pessah,§ and Susan L. Schantz†

University at Buffalo, Buffalo, New York 14214; †University of Illinois, Urbana, Illinois 61802; ‡College of Charleston, Charleston, South Carolina 29424; §University of California, Davis, California 95616; and [¶]Wadsworth Center, NYSDOH, Albany, New York 12201

Received August 8, 2005; accepted September 19, 2005

Each environmental exposure matrix contains a unique mixture of PCB congeners. Since several congener types have multiple and distinct biological actions, it is important to characterize congener profiles in exposure sources. The Fox River Environment and Diet Study (FRIENDS) is assessing the human health effects of consumption of PCB-contaminated fish from the Fox River in northeastern Wisconsin. Concurrent laboratory studies required the formulation of a dosing solution which closely mimicked the human PCB exposure from fish. PCB congener profiles from Fox River walleye were compared to profiles for various theoretical mixtures having different relative percentages of Aroclors by weight. The theoretical mixture which provided the best approximation of the Fox River fish PCB profile contained 35% 1242, 35% 1248, 15% 1254, and 15% 1260. A PCB mixture was formulated to match this theoretical construct, and the congener profile for the mixture of Aroclors was determined by capillary column gas chromatography with electron capture detection (GC/ECD). The relative percent of each congener was compared to the PCB congener profile of the theoretical Aroclor mixture and that for Fox River walleye. The specific congeners differed on average by 17% from the theoretical Aroclor mixture predicted values, and the specific congeners measured in the mixture were on average within 71% of those reported for Fox River fish. The mixture was found to have relatively low AhR activity but high RyR activity. Indirect comparisons suggest that *in vivo* toxicity was slightly greater than that for Aroclor 1254. This illustrates that Aroclor mixtures are useful for formulating dosing solutions which closely approximate actual environmental exposures.

Key Words: **PCB congeners; dosing solutions; Aroclor mixtures.**

It is well-recognized that individual PCB congeners or commercial formulations such as Aroclors do not reflect the composition of environmental exposure sources. Each environmental exposure matrix contains a unique mixture of PCB congeners. Because the several classes of congeners have multiple and distinct biological actions (Safe, 1994), it is important to characterize congener profiles in exposure sources and attempt to determine the biological actions of these various mixtures.

In a few cases, actual environmental mixtures of PCBs have been tested for toxicity in animal models. Animals have been exposed to PCB-contaminated soils (Fouchecourt *et al.*, 1998; Hansen *et al.*, 1981) or extracts of soils (Li and Hansen, 1996a,b). Feeding animals Great Lakes fish also closely models an important exposure source (Restum *et al.*, 1998; Shipp *et al.*, 1998) and is especially valuable in studies where the PCB composition of the fish has been determined in detail (Gerstenberger *et al.*, 2000; Jordan and Feeley, 1999).

Other studies have used congener-specific analyses of exposure sources to formulate PCB mixtures mimicking the congener profile in the source. For example, Rice and Hayward (1999) conducted a study in which infant monkeys were exposed to a mixture of 15 PCB congeners formulated to represent the congeners present in the breast milk of Canadian women. Similarly, Hany and colleagues (1999) exposed laboratory rats to an experimental mixture of 13 PCB congeners designed to be representative of the PCBs found in human breast milk. The advantage of creating an environmentally relevant PCB congener profile is a very well-defined mixture and a better understanding of the potential health effects for the affected populations, but simulated environmental mixtures prepared from individual congeners are very costly to prepare and can, at best, include a relatively small subset of the major congeners present in the environmental source.

Now that the various Aroclor formulations have been more accurately characterized (Frame *et al.*, 1996), more realistic mixtures, including a large number of major and minor congeners, can be formulated from these Aroclors. Once the PCB profile in the environmental matrix of interest is determined in detail, specific Aroclor profile data can be used to create mixtures containing different proportions of individual Aroclors that best approximate the target PCB profile. The current study was performed to formulate a dosing solution that mimicked the PCB congener profile in Fox River Fish that is

[1] To whom correspondence should be addressed at University at Buffalo-Toxicology Research Center, Hayes B, 3435 Main St., Buffalo, NY 14214. Fax: (716) 829-2806. E-mail: pjkost@buffalo.edu.

regularly being consumed by a study population in Green Bay, Wisconsin. The dosing solution was to be used in rodent laboratory studies assessing the neurodevelopmental and neurobehavioral effects of gestational and lactational exposure to the Fox River PCB congener profile. PCBs that have at least three chlorines in the lateral positions and zero or one chlorines in an ortho position can bind to the aryl hydrocarbon receptor (AhR) and exert at least some of their toxic effects through the AhR pathway. In contrast, PCBs with two or more ortho-substitutions have very low affinity for the AhR but do bind to ryanodine-sensitive calcium channels (RyR) and may exert some of their toxic effects by altering ryanodine-sensitive calcium signaling within cells. Therefore, toxicity of the mixture was characterized by assessing both its AhR activity via a luciferase reporter gene assay (Gasiewicz *et al.*, 1996) and its ryanodine receptor (RyR) channel activity using a ^{3}H-ryanodine binding assay (Pessah *et al.*, 1987). Toxicity was further characterized via an *in vivo* dose-response assessment of developmental toxicity in laboratory rats.

MATERIALS AND METHODS

Development of Aroclor target mixture to match Fox River Fish PCB congener profile. The PCB congener profiles for eight walleye collected from the Fox River were previously determined by Exponent Corporation in 1998 for the Wisconsin DNR (Paulson, personal communication). An average for each congener reported was calculated and normalized to determine the weight percent of that congener in the fish. The PCB profile for the Fox River walleye was presented graphically and compared to the profiles of four commercially manufactured Aroclors; 1242, 1248, 1254, and 1260 (Fig. 1). The PCB profile for the fish was found to differ considerably from the profiles of any individual commercial Aroclor.

Theoretical PCB congener profiles were generated based upon a mixture of four commercially manufactured Aroclors (1242, 1248, 1254, and 1260) with the goal of closely matching the mean PCB profile determined for the Fox River fish. The identification and composition (weight percent) of the PCB congeners in each commercial Aroclor to be used in formulating the theoretical mixture were characterized by Frame *et al.* (1996) for various lots of each Aroclor. The values used in the theoretical constructs used the means of several lots as follows: 35% Aroclor 1242 [mean of A3, G3, and S3B], 35% Aroclor 1248 [G3.5], 15% Aroclor 1254 [A4], and 15% Aroclor 1260 [mean of A5, S5, and G5]. The weight percents of the PCB congeners within each Aroclor were recalculated to include only the 91 PCB congeners that were determined in the Fox River fish. These recalculated normalized weight percent values were then multiplied by the percentage of each specific Aroclor in the formulated theoretical mixture. The total weight percent of each PCB congener in the theoretical mixture was based upon the sum of the contribution from each Aroclor. Theoretical mixtures using various proportions of the commercial Aroclors were then compared graphically to the mean PCB profile in Fox River walleye using Microsoft Excel. The theoretical PCB mixture that most closely approximated the PCB profile found in Fox River Fish was a mixture of 35% Aroclor 1242, 35% Aroclor 1248, 15% Aroclor 1254, and 15% Aroclor 1260.

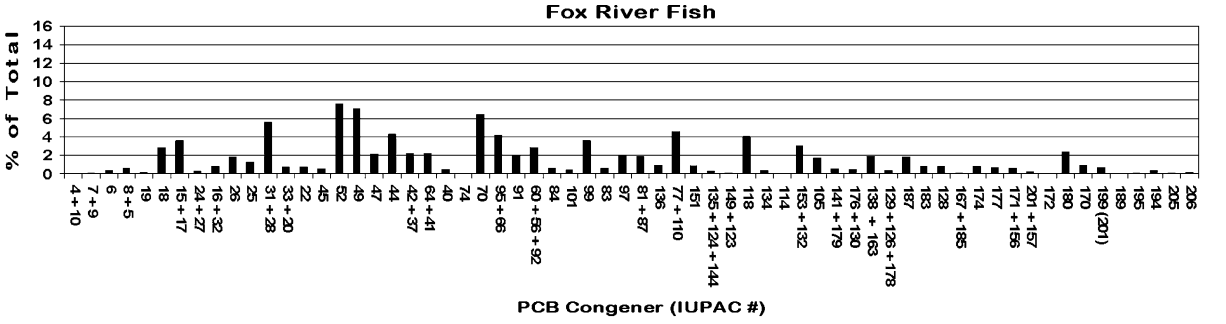
Formulating actual Aroclor mixtures to mimic theoretical mixtures presents many challenges. One cannot use pipets and other transfer utensils since the Aroclors, especially 1254 and 1260, are not transferred quantitatively owing to their physical properties. Aroclor 1260 is highly viscous with a waxy consistency. In order to overcome the 1260 transfer problem, the vials of 1248 were added to each of four vials of 1260, and sonicated in warm water (~60°C) to suspend the material. This permitted transfer of the major portion of the contents into a cleaned and tared vessel. The 1248 vials were rinsed with accurately weighed 1242. Each vial was heated to about 60°C and emptied with continued heating into the tared vessel. The final PCB mixture formulated to match the theoretical construct contained the following respective amounts of each Aroclor: 55.3g 1242 (Monsanto Lot KB 05–415), 56.4 g 1248 (AccuStandards Lot F-110), 25.4 g 1254 (Monsanto Lot KB 05–612), and 24.1 g 1260 (AccuStandards Lot 021–020). The mixture was diluted in corn oil, and duplicate samples were sent to the Core Analytical Toxicology facility at the Toxicology Research Center, State University of New York at Buffalo for analysis.

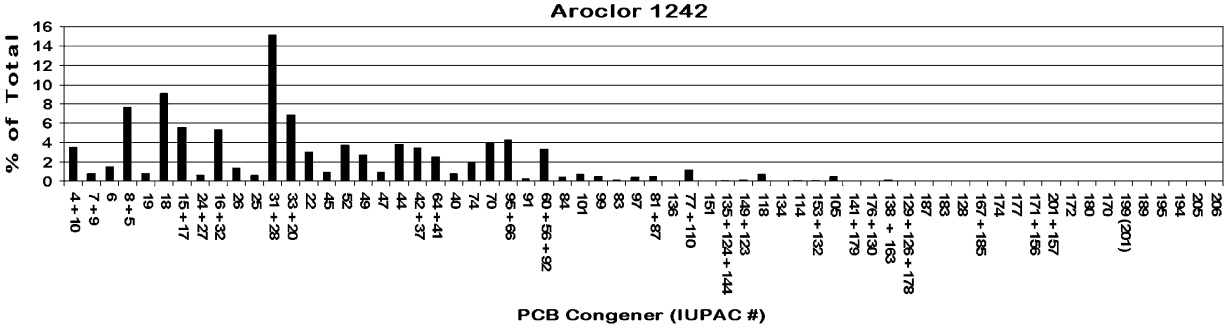
The Aroclor lots used in the mixture are not the same lots that were characterized by Frame *et al.* (1996). The lots for Aroclors 1242, 1248, and 1260 used in the mixture were not analyzed by us, but are expected to be similar to those characterized by Frame *et al.* (1996). The Aroclor 1254 lot used in the mixture was analyzed and determined to be indistinguishable from the 1254 A lot used by Frame *et al.* (1996). (Cochran, J., 1999, personal communication).

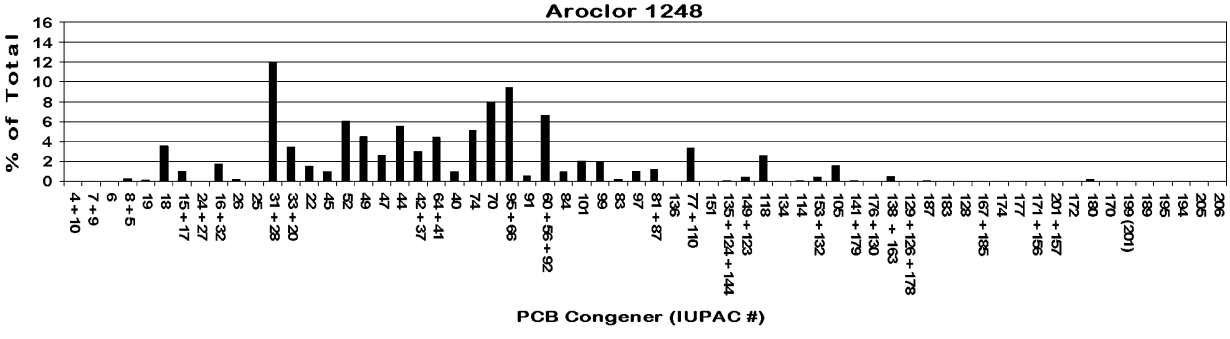
Determination of PCB congener profiles in the PCB mixture. Congener specific analysis of the actual Aroclor mixture was performed at the Core Analytical Toxicology Facility based upon the methods described by Greizerstein *et al.* (1997) and Howard *et al.* (2003). Three aliquots each of the two samples were weighed and analyzed in duplicate. The six samples were diluted in n-hexane, and congeners #30 and #204 were added as internal standards prior to analysis by capillary column gas chromatography with electron capture detection (GC/ECD). The Hewlett Packard 6890 gas chromatograph (Palo Alto, CA) was equipped with temperature and pressure programming capabilities and a split/splitless injector. A HP 7683 automated liquid sampler performed sample introduction. Separation of individual PCB congeners was performed using a 60-m SPB-5 fused-silica capillary column (0.25-mm i.d., 0.25-μm film thickness) from Supelco (Bellefonte, PA). The carrier gas was ultrapure helium at an initial head pressure of 34 psi and constant flow rate of 1.5 ml/min. The detector make-up gas was ultrapure nitrogen at a flow rate of 60 ml/min. A sample volume of 1.0 μl was injected into the splitless injector, operated in the splitless mode (0.75 min splitless vent time, 100 ml/min) at a temperature of 260°C. The detector temperature was 300°C. The oven temperature program started at 130°C, programmed to 200°C at 4°C/min, then to 210°C at 1.0°C/min, and finally to 280°C at 2.0°C/min, for 5 min.

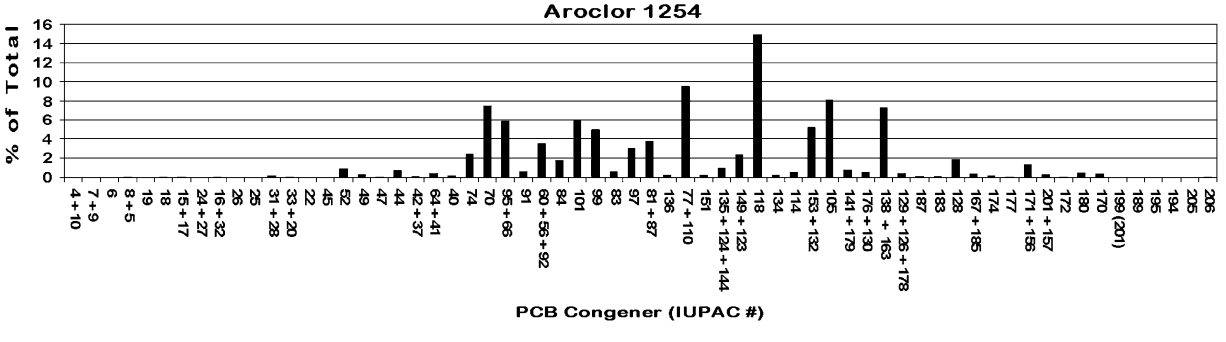
The GC data were acquired and quantified using Perkin-Elmer TurboChrom software. Identification and quantitation of the individual congeners were performed by comparison with reference standards. The analytes were identified by their retention times relative to the respective internal standards (congener #30 for peaks eluting before congener #101, and congener #204 for congener #101, and those eluting thereafter). Calibration curves (second-order polynomial) were generated based upon dilutions of an EPA PCB Congener Calibration Check solution (Ultra Scientific No. RPC-EPA) for quantitation of congeners 8, 18, 28, 44, 52, 66, 77, 101, 105, 118, 126, 128, 138, 153, 170, 180, 187, 195, and 206. The concentrations of congeners not present in the EPA calibration standard were calculated using average response factors generated in our laboratory, determined from individual congener reference standards. The results from the GC analysis were exported to an Excel spreadsheet where the weight percent distribution of individual congeners was calculated as a percentage of the total PCBs determined. These results from the actual Aroclor mixture were then compared to the theoretical Aroclor mixture and the Fox River fish PCB profile.

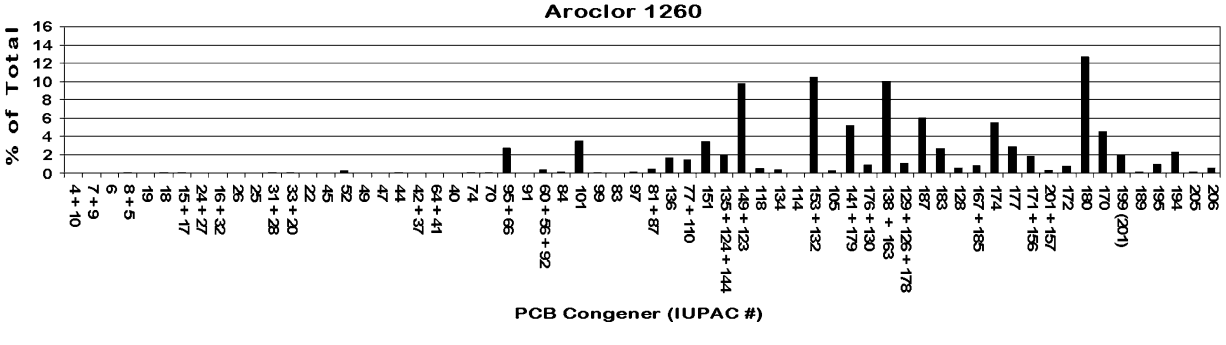
Luciferase reporter gene bioassay for dioxin-like activity. The reporter gene bioassay system is based on the premise that dioxin-like compounds including certain PCBs initially bind to the AhR, and subsequently the ligand-activated AhR binds to dioxin response elements on DNA. A murine hepatoma cell line (hepa 1c 1c7) was stably transfected with the p2Dluc plasmid containing two DREs upstream from the firefly luciferase gene. A 96-well cell culture plate format has been optimized for this cell-based reporter gene bioassay system to assess AhR-responsive luciferase activity of dioxin-like chemicals, individually and in complex, real-world mixtures. The reporter gene bioassay is exceptionally sensitive, detecting levels of TCDD as low as 1 pM


Fox River Fish
% of Total
PCB Congener (IUPAC #)


Aroclor 1242
% of Total
PCB Congener (IUPAC #)


Aroclor 1248
% of Total
PCB Congener (IUPAC #)


Aroclor 1254
% of Total
PCB Congener (IUPAC #)


Aroclor 1260
% of Total
PCB Congener (IUPAC #)

(0.06 pg/200 µl/well). Murine hepatoma 1c1c7 cells, stably transfected with the p2Dluc plasmid were obtained from Dr. T. A. Gasiewicz, University of Rochester (Gasiewicz *et al.* 1996). Cells were maintained in Eagle's modified MEM (Invitrogen Life Technologies) supplemented with 10% FBS, sodium bicarbonate (1.5 g/l), sodium pyruvate (10 ml/l), nonessential amino acids, and an antibiotic/antimycotic solution. Cells were maintained in a humidified atmosphere containing 5% $C0_2$ and were routinely passaged by trypsinization. For each experiment, opaque 96-well culture plates were seeded with 100,000 cells/well, and cells were allowed to adhere for 24 h prior to treatment. Upon treatment, cells were treated with 1% DMSO (control) or sample dissolved in DMSO. Cells were treated for 18 h, and the luciferase activity of sample was analyzed by luminometry.

^{3}H-Ryanodine binding analysis ryanodine receptor channel activation. The binding of ^{3}H-ryanodine to its high-affinity sites on junctional microsomal membranes enriched in type 1 ryanodine receptor (RyR1) was performed as previously described (Wong and Pessah, 1996). Microsomal membranes were isolated from rabbit skeletal muscle by sucrose density gradient and stored at −80°C until needed (Pessah *et al.*, 1987). The radioligand receptor binding assay consisted of 12.5 µg of microsomal protein and 1 nM ^{3}H-ryanodine (specific activity 57 Ci/mmol) in a buffer consisting of (in mM) NaCl (15), KCl (140), HEPES (20), Ca^{2+} (0.05) in a final volume of 500 µl. Each PCB mixture was titrated in the assay by addition of 5 µl from a 100× stock in DMSO. An equivalent volume of DMSO was added to controls. The binding reactions were incubated for 3 h at 37°C, at which time the reactions were quenched by rapid filtration through GF/B glass fiber filters using a 24-sample cell harvester (Brandel). Nonpecific binding was determined in the presence of 1000-fold excess unlabeled ryanodine and was not influenced by the highest concentration of PCB used in the study. The molar concentration of each mixture was based on an average molecular weight of each mixture (A1242 = 257; A1248 = 292; A1254 = 326; A1260 = 366, and the theoretical Aroclor mixture = 296).

The same Aroclor lots used in the mixture were used in this analysis. The EC_{50} and maximum specific binding (B_{max}) obtained with each mixture were calculated from a fit of the dose-response relationships to a sigmoidal equation (Origin).

Developmental toxicity in rats. The developmental toxicity of the mixture was assessed *in vivo* by exposing female Long-Evans rats (Harlan, Madison, WI) and measuring developmental endpoints including maternal weight gain, gestation length, litter size, percent live births, birth weight, and postnatal growth. Adult female rats were dosed orally with 0, 1, 3 or 6 mg/kg/day of the experimental mixture beginning 4 weeks before they were bred and continuing through postnatal day 21. The PCB congener concentrations and percent compositions present in the experimental mixture are given in Table 1. The PCB mixture was diluted in corn oil and pipetted onto one half of a vanilla wafer cookie (Keebler Golden Vanilla Wafers®), and the treated cookies were fed to the females daily throughout the exposure period. The dose was adjusted daily to account for weight gain of the dams.

After 4 weeks of exposure, the females were bred with unexposed males. For breeding, each female was paired daily with an unexposed male for a maximum of 8 days. Pregnancy was determined by the appearance of a sperm plug, at which point the pregnant females were separated from the males and housed individually. On PND 2 (day of birth = PND 0), the litters were reduced to 10 pups (five males and five females where possible), cross-fostering with extra pups from the same treatment group as needed. Pups were weighed at birth and on postnatal days 7, 14, and 21. Two males and two females from each litter were saved for behavioral testing, and when extra pups were available, one male and one female per litter were euthanized on postnatal day 21, and liver, thymus, and brain weights were obtained for comparison to body weight. The animal facility was AAALAC approved and all procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Illinois.

TABLE 1
Percent PCB Congener Composition and Concentration in Dosing Solution

PCB congener[a]	% of total[c]	Conc. (µg/g)[d]	PCB congener[a]	% of total[c]	Conc. (µg/g)[d]
4 + 10	1.51	650.0	77 + 110	3.76	1622.7
7 + 9	0.34	145.4	82*	0.39	169.0
6	0.53	230.2	151	0.82	352.5
8 + 5	2.87	1238.4	135 + 124 + 144	0.49	210.5
19	0.53	227.1	147 + 109*	0.18	77.6
18	4.38	1890.5	149 + 123	1.50	649.2
15 + 17	2.80	1210.0	118	2.86	1233.0
24 + 27	0.26	111.6	134	0.11	46.1
16 + 32	2.69	1160.1	114	0.10	43.2
26	0.69	298.8	188 + 146*	0.31	132.2
25	0.27	118.3	153 + 132	2.54	1097.3
31 + 28	7.69	3320.4	105	2.05	885.0
33 + 20	3.30	1425.4	141 + 179	0.89	384.2
51*	0.27	117.3	176 + 130	0.24	104.3
22	1.29	557.7	138 + 163	2.37	1021.5
45	0.82	355.2	158*	0.47	203.0
52	3.89	1677.7	129 + 126 + 178	0.28	119.9
49	2.49	1077.1	187	0.72	312.4
47	1.00	430.7	183	0.45	194.1
48*	0.84	364.4	128	0.40	174.3
44	3.29	1421.5	167 + 185	0.25	107.7
59*	0.27	116.2	174	0.79	339.4
42 + 37	1.69	730.8	177	0.47	203.1
71*	0.77	331.3	171 + 156	0.41	179.1
64 + 41	2.48	1071.9	201(200)[b] + 157	0.10	43.6
40	0.72	310.9	172	0.15	63.1
74	2.20	950.2	180	1.49	642.3
70	4.25	1834.8	191*	0.03	12.2
95 + 66	5.63	2429.3	170	0.73	315.3
91	0.50	214.8	190	0.15	66.7
56 + 60 + 92	4.63	2000.4	199 (201)[b]	0.34	146.0
84	0.96	414.3	203 + 196*	0.37	158.7
101	2.96	1276.0	189	0.03	12.9
99	1.61	694.9	195	0.14	58.7
83	0.26	113.1	194	0.42	182.8
97	1.04	451.1	205	0.02	9.3
81 + 87	1.06	458.0	206	0.08	35.0
136	0.32	137.7			

[a]PCBs identified by IUPAC numbers.
[b]PCB identified by BZ#.
[c]Percent of congener present in the actual Aroclor mixture.
[d]Concentration (µg/g) of PCB in dosing solution.
*PCB congeners not reported in Fox River fish, but present in dosing solution.

FIG. 1. PCB profiles for Fox River fish compared to individual previously available commercial Aroclors. The mean PCB congener profiles for eight walleye taken from the Fox River are presented in the upper panel. The four lower panels contain the profiles for actual Aroclors (Frame *et al.* 1996) normalized to the specific congeners in fish and presented on a weight percent basis.

RESULTS

The PCB profile found in Fox River fish is presented in Figure 1. The relative amount of each congener is expressed as the percent of total PCB measured in the sample (sum of all congeners). The relative amounts of each of the congeners present in Fox River fish were calculated for each of four Aroclors (1242, 1248, 1254, and 1260), and these profiles are presented in separate panels in the same figure. None of the individual Aroclors provided a good approximation of the profile found in the fish, in spite of the fact that the majority of the environmental contamination of the river resulted from Aroclor 1242. A mixture of all four Aroclors was required in order to duplicate the congener profile found in the Fox River fish.

The theoretical Aroclor mixture giving the closest match to the PCB profile in Fox River fish consisted of 35% Aroclor 1242, 35% Aroclor 1248, 15% Aroclor 1254, and 15% Aroclor 1260. This was designated as the theoretical Aroclor mixture. The PCB profile for the theoretical mixture is plotted next to the congener profile found in the fish in Figure 2. Relative differences calculated for each congener and expressed as a percent are provided in the lower panel of the same figure. The relative percent difference (RPD) is calculated using the formula $RPD = (X_1 - X_2)/(1/2(X_2 + X_1)) \times 100$. The congener value for the fish was, on average, within 72% of the Aroclor mixture values.

The actual mixture of PCBs made from the four Aroclors contained 34.5% A1242, 35.2% A1248, 15.9%, A1254, and 15% A1260. The actual PCB concentrations present in the

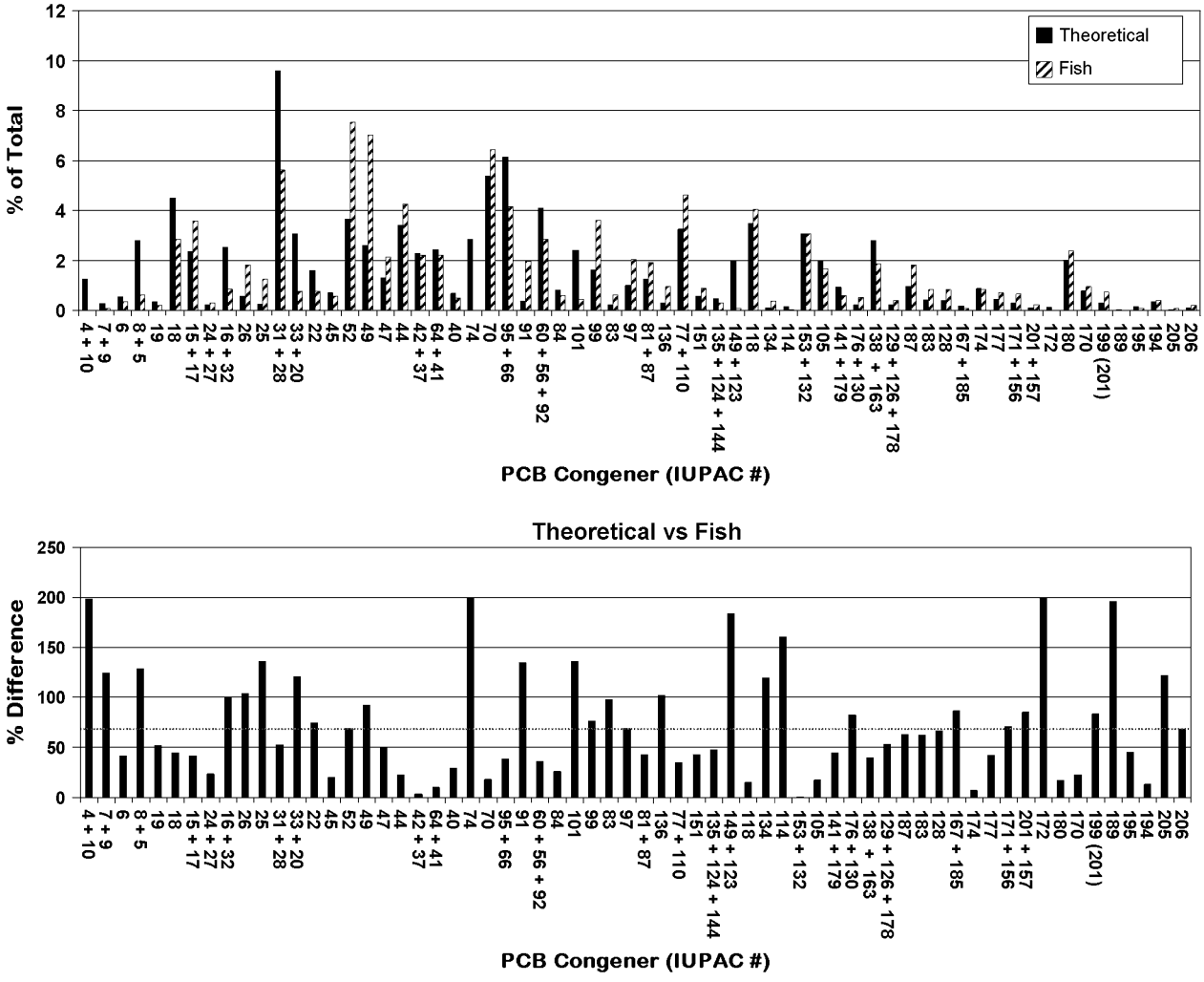


FIG. 2. PCB profiles for the theoretical mixture construct which best reflected PCB profiles in Fox River fish. The theoretical PCB mixture construct which gave the closest approximation of the profile found in Fox River Fish was a mixture of 35% Aroclor 1242 (Monsanto Lot KB 05–415, received May 1972), 35% Aroclor 1248 (AccuStandards Lot F-110), 15% Aroclor 1254 (Monsanto Lot KB 05–612, received May 1972) and 15% Aroclor 1260 (AccuStandards Lot 021–020). The congener profile for the theoretical PCB mixture construct is compared to the PCB congener profile for Fox River walleye in the upper panel. The lower panel provides the relative percent difference (RPD) between the theoretical mixture construct values versus the Fox River walleye values for each congener or group of congeners. The dashed line indicates the mean RPD of 72%.

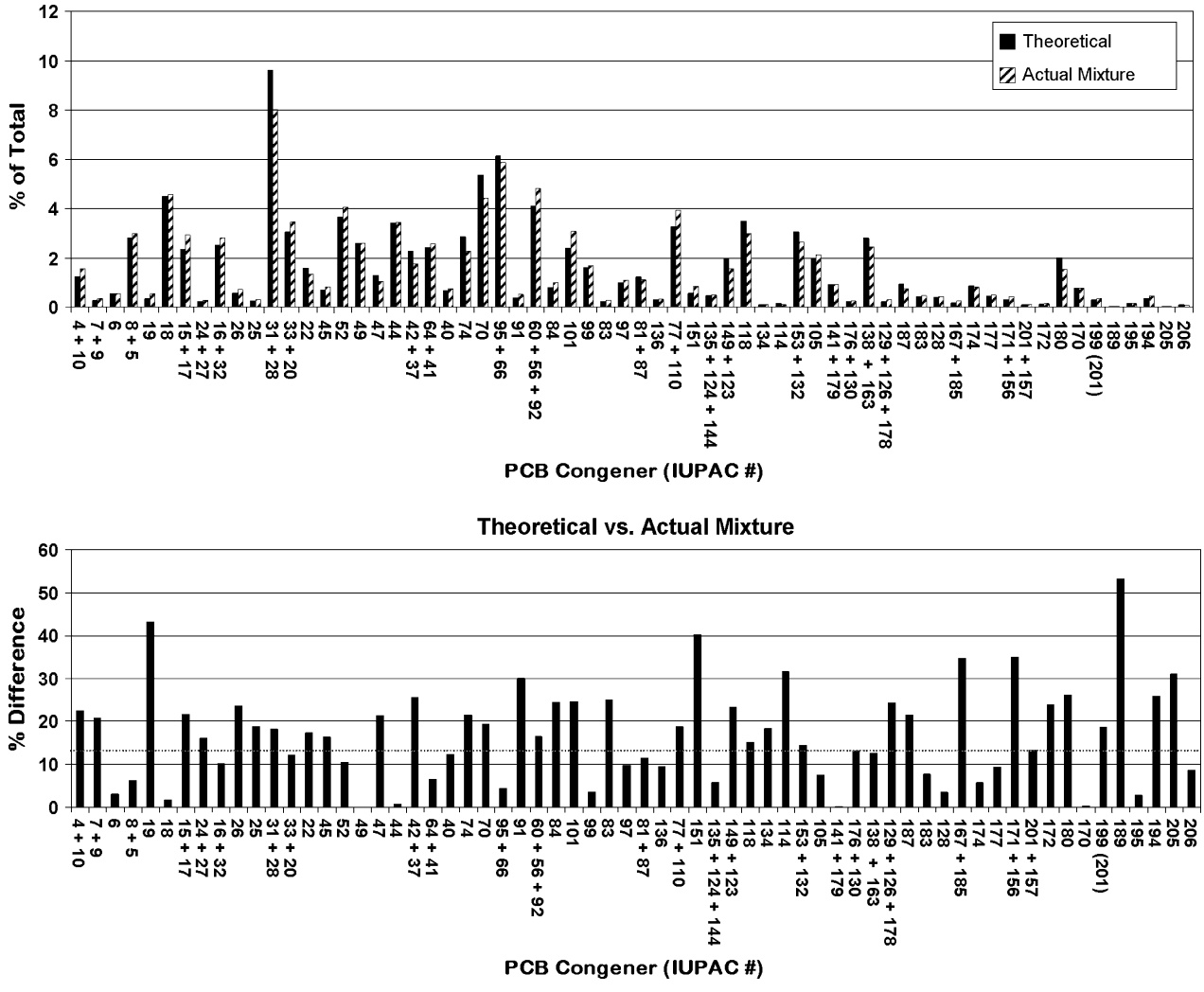


FIG. 3. The actual Aroclor mixture PCB profile compared to the theoretical PCB mixture construct. The congener profile for the actual Aroclor mixture, determined by GC-ECD, is compared to the PCB congener profile of the theoretical Aroclor mixture construct that best reflected the mean PCB congener profile for Fox River walleye in the upper panel. The lower panel provides the relative percent difference (RPD) between the actual Aroclor mixture values versus the theoretical mixture construct values for each congener or group of congeners. The dashed line indicates the mean RPD of 17%.

mixture are given in Table 1. This actual mixture was analyzed for individual PCB congeners, and the congener profile was compared to the theoretical mixture in Figure 3. The actual PCB mixture profile was very similar to the expected profile. The specific congeners measured were, on average, within 17% of the predicted values. The actual Aroclor mixture PCB profile was then compared to the profile of PCBs in Fox River fish (Fig. 4). The specific congeners measured were, on average, within 71% of the values found in Fox River fish.

The dioxin-like activity in the PCB mixture was estimated by the cell-based reporter gene bioassay system that assesses AhR-responsive luciferase activity of dioxin-like chemicals. Table 2 presents results from the reporter gene bioassay and from a theoretical calculation of dioxin toxic equivalents (TEQs). The theoretical TEQs were calculated based on the relative percent weights reported by Frame *et al.* (1996), Rushneck *et al.* (2004), and Takasuga *et al.* (in press) for the following coplanar and mono-ortho PCBs present in the mixture: 77, 81, 118, 114, 105, 123, 126, 156, 157, 167, and 189. The relative percent weights were used to determine the mass of each congener, and the TEQ calculation represents the sum of the products of the mass of the coplanar or mono-ortho PCBs and the respective toxic equivalency factor (TEF) for a given congener (Van den Berg *et al.*, 1998). A TEQ was not calculated for the Fox River fish, since there was limited data for coplanar and mono-ortho PCBs, and six of the coplanar PCBs are coeluters and, therefore, could not be calculated directly for use in the TEQ calculation. The bioassay and theoretically calculated dioxin-like activity in the PCB mixture were expressed as pg TEQ per mg of PCBs. The bioassay based estimate of TEQs (16–35 pg/mg PCB) was higher than the theoretically calculated value (2.7–3.2 pg/mg PCB).

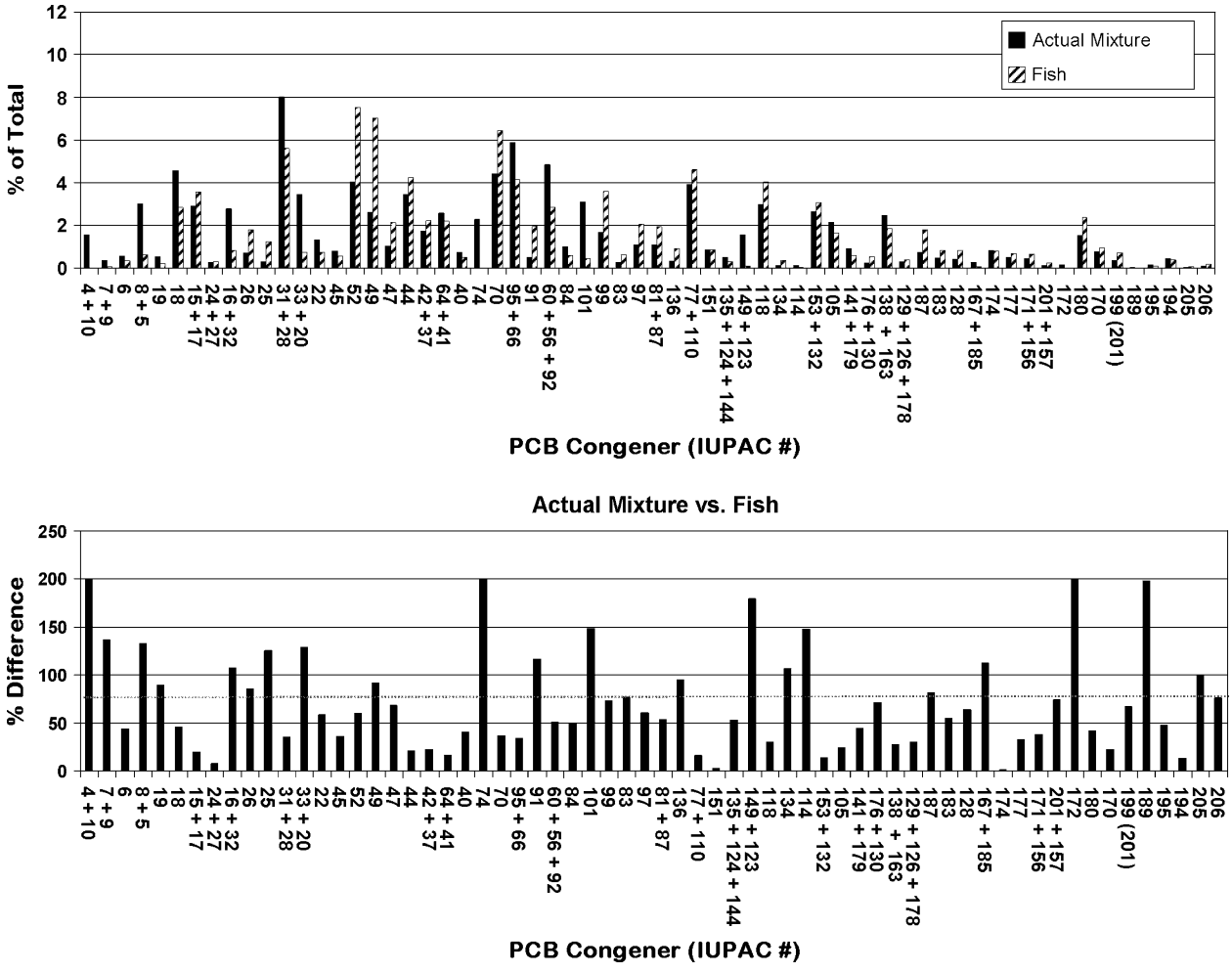


FIG. 4. The actual Aroclor mixture PCB profile compared to the PCB profile in Fox River walleye. The congener profile for the actual Aroclor mixture, determined by GC-ECD, is compared to the PCB congener profile for Fox River walleye ($n = 8$) in the upper panel. The lower panel provides the relative percent difference (RPD) between the actual Aroclor mixture values versus the Fox River walleye values for each congener or group of congeners. The dashed line indicates the mean RPD of 71%.

The radioligand ^{3}H-ryanodine binds to RyR1 with high affinity when the channel is in an open conformation (Pessah *et al.*, 1987), and this conformational sensitivity of ^{3}H-ryanodine binding was used to quantitatively assess the dose response relationship between the Fox River mixture and selected Aroclors as previously described by Wong and Pessah (1996). The dose-response curve of each mixture is shown in Figure 5. Compared to the Aroclors from which it was derived, the PCB mixture created to simulate the congener distribution found in Fox River fish possessed an intermediate activity toward activating RyR1 channels. Specifically, the PCB mixture showed a potency (EC_{50}) closest to A1248, and a maximal efficacy equivalent to A1242. The slope of the dose-response obtained with the PCB mixture was steeper than that observed with Aroclors, suggesting a higher proportion of RyR-active congeners.

There were no overt signs of clinical toxicity in the dams from any of the treatment groups. Gestational weight gain, lactational weight gain, liver weight, litter size, percent live births, and percent male pups were analyzed using ANOVA and determined to be similar between the PCB-exposed and control rats (Table 3). An ANOVA analysis determining if litter size changed significantly with dose just missed significance ($p = 0.052$). Overall, the pups in all three exposure groups weighed less than control pups at birth, although the weight differences were not significant for the male pups in the 3 mg/kg exposure group (Table 4). Pups in the highest exposure group (6 mg/kg/day) weighed about 10% less than control pups at birth. This reduction in offspring weight for the exposed pups was also evident at weaning. The offspring in the 6 mg/kg group exhibited slower postnatal growth, resulting in body weights that were approximately 19% less than the control pups at weaning. Liver-to-body-weight ratios were significantly increased in pups from all three exposure groups, with the ratio nearly doubling in the 6 mg/kg/day group (Table 5). In contrast, the brain-to-body-weight ratio was not altered in any of the

TABLE 2
Dioxin-Like Activity in the Aroclor Mixture

	pg TEQ/mg PCB
Theoretical TEQs[a]	
Frame *et al.*, 1996	2.7
Rushneck *et al.*, 2004	3.2
Takasuga *et al.*, 2005	2.5
Bioassay measured TEQs[b]	
Average	25
Range	16–35

[a]Calculated based on percent weights from cited literature and TEFs from WHO (Van den Berg *et al.*, 1998).
[b]Based on results from luciferase assay.

exposure groups, and the thymus-to-body-weight ratio was significantly reduced only in the female pups from the 6 mg/kg/day group. Weight gains were analyzed using repeated measures of ANOVA, and significant effects were followed by Dunnett's tests to determine which treatment group differed from controls.

There was no exposure-related mortality among the pups; however, it was observed that seven of the eleven pups in one litter from the 1 mg/kg/day exposure group were killed by the dam on postnatal day 2.

DISCUSSION

The PCB profiles in Fox River fish are considerably different from Aroclor 1242, which is the primary source of contamination of that river (Imamoglu *et al.*, 2004). Similarly, the PCB congener profiles of the higher chlorinated Aroclors 1248, 1254, and 1260 indicate that they may be a source for some additional congeners present in the fish, but their PCB profiles also do not mimic closely the PCB profile seen in the fish. This is not an unexpected finding. The differences observed in Aroclor PCB profiles versus fish PCB profiles could be due to a number of factors. Aroclors deposited in the river can undergo congener-specific biotransformation in sediments (Magar *et al.*, 2005). Specific congeners have different physical-chemical properties, which can result in congener-specific differences in partitioning into the water column and into the aquatic food chain (Czub and Mclachlan, 2004). Similarly, metabolism and excretion rates vary among different congeners, and this accounts for an enhanced retention of congeners with higher degrees of chlorination, such as 153, 138, 180, and 118 (Safe, 1994). Thus, one would not expect to find a PCB profile in fish that closely reflects that of a specific Aroclor.

The use of individual congeners to formulate PCB dosing solutions which mimic environmental exposures would require large quantities of individual congeners. This approach is expensive for the quantities of dosing solution required for long-term studies. Depending on the effects being studied,

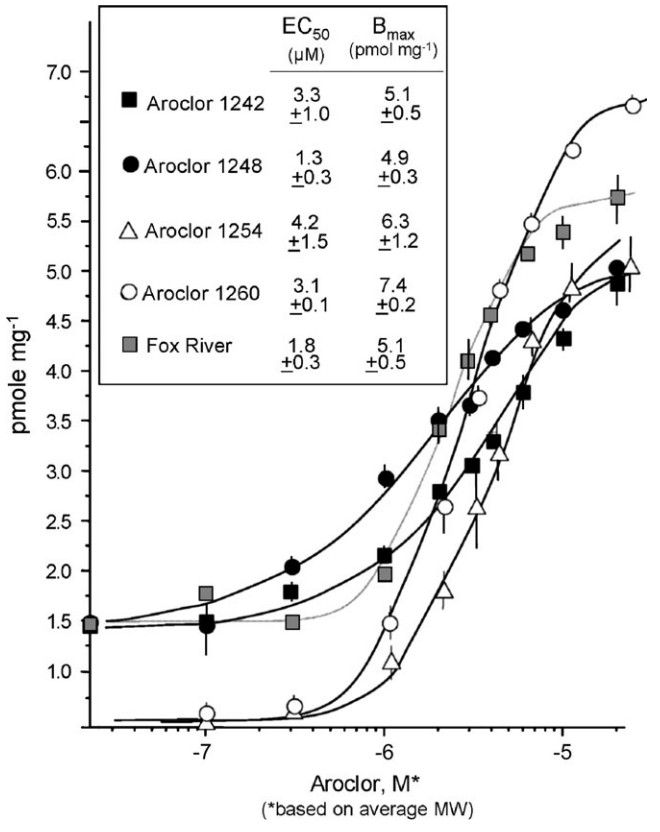


FIG. 5. Dose-response relationship obtained from the PCB mixture simulating the congener profile found in Fox River walleye (Fox River) and four Aroclors toward activation of Ca^{2+} channel RyR1. ^{3}H-Ryanodine-binding analysis was performed under steady-state conditions as described in Methods. The molar concentration was calculated from the average molecular weight calculated for each mixture (see methods for details). The potency (EC_{50}) and efficacy (B_{max}) and associated SD were calculated from at least triplicate determinations.

some congeners may be agonists, others antagonists with different potencies, thus inclusion of as many relevant congeners as possible in proportions similar to those in environmental samples is important. Although this study focuses on PCBs, there are other chemicals, such as pesticides and metals, present in fish that should be considered when assessing potential health risks from consuming contaminated fish. However, assessing chemicals other than PCBs was beyond the scope of this study.

The approach of using Aroclor mixtures has several advantages: specific Aroclors have been well characterized with respect to congener profiles (Frame *et al.*, 1996); these mixtures are available in the quantities necessary to prepare sufficient material for long-term animal studies; the cost of the Aroclors is not prohibitive; and the Aroclors can be mixed in the laboratory using readily available equipment. It is important to note that PCB profiles in Aroclors can differ between lot numbers, which is most evident between the 1254 A and 1254 G lots, which have major differences in congener percent weights. (Frame *et al.*, 1996). The 1254 lot used in this study

TABLE 3
Reproductive Outcomes of Dams Exposed to 0, 1, 3, or 6 mg/kg/day PCB (Fox River Mix) during Gestation and Lactation

PCB dose	Dam gestational weight gain (g)	Dam lactational weight gain (g)	Dam liver weight (g)	Litter size	% Male	% Live births
0 mg/kg/day (control; $n = 13$)	111.92 ± 5.82	15.69 ± 4.96	13.50 ± 0.51	9.69 ± 0.75	42.58 ± 4.23	100 ± 0.00
1 mg/kg/day ($n = 14$)	112.07 ± 4.21	16.83 ± 3.99	12.69 ± 0.49	11.18 ± 0.70	58.79 ± 3.75	100 ± 0.00
3 mg/kg/day ($n = 10$)	106.20 ± 5.12	28.10 ± 3.77	13.86 ± 0.30	9.30 ± 0.83	47.23 ± 4.45	100 ± 0.00
6 mg/kg/day ($n = 11$)	100.09 ± 4.79	20.64 ± 4.57	14.41 ± 0.52	7.82 ± 0.87	50.05 ± 6.28	100 ± 0.00

Note. Values represent the mean ± SEM; *n* represents the number of dams. Litter size is the average litter size at birth.

is similar to the 1254 A lot characterized by Frame *et al.* (1996). The 1254 A lot has been reported to have high TEQ activity, whereas the 1254 G lot has been reported to have a low TEQ activity (Frame, 1999).

The actual mixture of Aroclors developed for this study has a congener distribution profile very similar to that seen in the Fox River fish. However, several notable differences do exist. Congeners found in the Aroclor Mix but not found in Fox River Fish include congeners 48, 51, 59, 71, 82, 109 + 147, 46 + 188, 158, 191, and 196+203. Additionally, compared to the Fox River fish congener profile, PCB congeners 4 + 10, 7 + 9, 8 + 5, 16 + 32, 33 + 20, 74, 101, 114, 149 + 123, 167 + 185, 172, and 189 are overrepresented, and congeners 25, 91, 134, and 205 are underrepresented. One would not expect the PCB profiles in fish to exactly mimic those seen in individual Aroclors. Some of the differences between fish and mixture profiles result from the fact that the Aroclors contain a wide variety of congeners, which in the environment undergo different rates of bioaccumulation and biotransformation within the aquatic food chain. Additionally since the Fox River fish analysis was performed by another laboratory using different analytical methods than used for the Aroclor mixture, these analytical differences may contribute to the fish and mixture profile differences. Future studies are planned to compare the toxicity of PCB congener extracts from Fox River fish to the toxicity in the PCB mixture.

Most of the toxicity studies in animal models have focused on individual PCB congeners or commercial PCB mixtures. However, as outlined above, the original commercial PCB mixtures do not accurately represent the actual PCB mixtures present in environmental matrices. When PCBs are present in complex mixtures, they may interact in complex ways to produce effects that are not readily apparent from specific congener studies. Therefore, animal toxicity studies using complex PCB mixtures formulated to mimic actual human exposure as closely as possible are critical in order to understand the health risks in human populations. The experimental mixture described in this paper closely models the PCB congener profile in contaminated fish being consumed by subsistence fishermen and their families in northeastern Wisconsin. Use of this mixture in animal toxicity studies will greatly facilitate our understanding of the potential health risks in humans eating the contaminated fish. Initial laboratory studies to characterize the general toxicity of the mixture included a reporter gene bioassay for dioxin-like activity, a ryanodine binding assay to identify the potency of the

TABLE 4
Average body weight (±SEM) for pups born to dams exposed to PCB (Fox River Mix) during gestation and lactation

	PND 0	PND 7	PND 14	PND 21
0 mg/kg/day PCB (control; 10 litters)				
MALE	6.81 ± 0.13	16.99 ± 0.54	30.82 ± 1.13	48.85 ± 1.87
FEMALE	6.50 ± 0.13	16.57 ± 0.56	30.14 ± 1.10	47.03 ± 1.49
1 mg/kg/day PCB (11 litters)				
MALE	6.44 ± 0.18[c]	16.40 ± 0.36	30.11 ± 0.72	46.23 ± 1.44[a]
FEMALE	6.10 ± 0.19[c]	15.64 ± 0.48[a]	28.81 ± 0.80	43.58 ± 1.41[b]
3 mg/kg/day PCB (9 litters)				
MALE	6.67 ± 0.12	16.47 ± 0.32	30.08 ± 0.59	47.49 ± 1.21
FEMALE	6.32 ± 0.11[a]	15.71 ± 0.36[b]	28.50 ± 0.72[b]	44.58 ± 1.12[b]
6 mg/kg/day PCB (6 litters)				
MALE	6.13 ± 0.07[c]	13.58 ± 0.75[c]	25.47 ± 1.30[c]	39.16 ± 2.19[c]
FEMALE	5.81 ± 0.08[c]	13.37 ± 0.37[c]	25.37 ± 0.65[c]	38.48 ± 1.42[c]

Note. Average body weight (±SEM) for pups born to dams exposed to 0, 1, 3, or 6 mg/kg/day PCB (Fox River Mix) during gestation and lactation Body weights are in grams. Each litter was standardized to 10 pups on PND 2.

[a]Group differs from controls at $p < 0.05$.

[b]Group differs from controls at $p < 0.01$.

[c]Group differs from controls at $p < 0.001$.

TABLE 5
Organ:body weight ratios on PND 21 for pups exposed to PCB (Fox River Mix) during gestation and lactation

	Brain:body weight ratio	Liver:body weight ratio	Thymus:body weight ratio
0 mg/kg/day PCB (Control)			
MALE ($n = 9$)	0.0300 ± 0.0012	0.0373 ± 0.0013	0.0037 ± 0.0003
FEMALE ($n = 10$)	0.0301 ± 0.0010	0.0357 ± 0.0004	0.0045 ± 0.0001
1 mg/kg/day PCB			
MALE ($n = 11$)	0.0324 ± 0.0008	0.0450 ± 0.0012[b]	0.0041 ± 0.0001
FEMALE ($n = 10$)	0.0330 ± 0.0013	0.0449 ± 0.0007[b]	0.0043 ± 0.0001
3 mg/kg/day PCB			
MALE ($n = 9$)	0.0314 ± 0.0006	0.0598 ± 0.0009[b]	0.0038 ± 0.0001
FEMALE ($n = 8$)	0.0317 ± 0.0009	0.0590 ± 0.0013[b]	0.0040 ± 0.0002
6 mg/kg/day PCB			
MALE ($n = 5$)	0.0340 ± 0.0015	0.0678 ± 0.0015[b]	0.0033 ± 0.0002
FEMALE ($n = 6$)	0.0330 ± 0.0016	0.0631 ± 0.0021[b]	0.0035 ± 0.0002[a]

Note. Organ:body weight ratios on PND 21 for pups exposed to 0, 1, 3, or 6 mg/kg/day PCB (Fox River Mix) during gestation and lactation. Values represent the mean ± SEM.

[a]Group differs from controls at $p < 0.01$.

[b]Group differs from controls at $p < 0.001$.

mixture in altering ryanodine sensitive calcium signaling and *in vivo* developmental toxicity testing in a rodent model.

TEQ Bioassay

Table 2 summarizes estimates of dioxin-like activity in the PCB mixture (pg TEQs/mg PCB) based on a theoretical calculation and from a cell-based AhR reporter gene bioassay. The calculated estimates are based on the reported percent by weight of dioxin-like coplanar and mono-ortho PCBs in the PCB mixture (Frame *et al.*, 1996; Rushneck *et al.*, 2004; Takasuga *et al.*, in press). Differences between the calculated and bioassay results could be due to higher levels of the dioxin-like PCBs in the actual PCB mixture and/or underestimated relative potencies (TEFs) for this bioassay. TEFs are rough estimates of toxic potency based on all available *in vivo* and *in vitro* data and not based on a specific bioassay (Van den Berg *et al.*, 1998). A potentially more likely explanation is that the reporter gene bioassay was trying to detect a very low dioxin-like TEQ activity in a relatively high level of PCBs. The very low TEQ activity in the PCB mixture (25 ppb) gave a response just above the limit of detection. Relatively high levels of non-dioxin-like PCBs were present in the bioassay and could have interfered with the reporter bioassay. Of greatest importance is the finding that the dioxin-like activity, in terms of TEQs, is very low in this PCB dosing mixture (about 25 ppb or 25 pg TEQs/mg PCBs) and thus has a very small toxicological contribution to the dosing solution.

RyR1 Bioassay

Ryanodine-sensitive Ca^{2+} channels (also known as ryanodine receptors; RyRs) are broadly expressed in both excitable and nonexcitable cells. RyRs are localized to discrete regions of the endoplasmic and sarcoplasmic reticulum (ER/SR), where they function as ion channels that regulate the release of Ca^{2+} from intracellular stores (Pessah and Wong, 2001). Aroclors and individual PCB congeners have been shown to potently sensitize the activation of RyRs by endogenous ligands (Wong and Pessah, 1996; Wong *et al.*, 1997). The structure-activity relationship for PCBs indicates that non-coplanar congeners with two or three *ortho*-chlorines are most active towards RyRs. The PCB mixture formulated to simulate the composition found in Fox River fish has a potency (EC_{50}) and efficacy (B_{max}) most similar to A1248 and A1242, respectively (Fig. 5). In this regard the B_{max} for the PCB mixture is intermediate when compared to the Aroclors from which it was derived, suggesting an additive D-R relationship among active congeners at RyR1. Other than the ryanodine studies, no direct comparison of the toxicity of the PCB congener mixture with any Aroclor mixture was performed.

In Vivo *Toxicity*

The *in vivo* developmental toxicity testing revealed developmental effects of similar or slightly greater magnitude to those we have observed previously with Aroclor 1254 (Roegge *et al.*, 2004). The study by Roegge *et al.* (2004) used the same PCB dose, dosing period, dosing method, and rat strain as was used in this study. Rat pups whose dams were exposed to 6 mg/kg/day Aroclor 1254 according to the same dosing protocol used in this study were not significantly smaller than control pups at birth, but did weigh approximately 15% less than control pups at weaning (Roegge *et al.*, 2004). In contrast, pups exposed to 6 mg/kg/day of the Fox River PCB mixture in the current study weighed about 10% less than control pups at birth and 19% less at weaning, suggesting a slightly larger impact on growth. Liver-to-body-weight ratios in the 6 mg/kg/day Aroclor 1254-exposed pups were increased to about the same

extent as those of pups exposed to 6 mg/kg/day of the Fox River PCB mixture, suggesting a similar degree of enzyme induction with the two mixtures (Roegge *et al.*, 2004). In contrast, thymus weights were impacted less by the Fox River PCB mixture than by a similar dose of Aroclor 1254 (Roegge *et al.*, 2004). Thymic atrophy is generally accepted as an AhR-mediated response, so this result is in line with the very low AhR activity of the Fox River PCB mixture, as measured in the luciferase reporter gene assay. In the future, this mixture will be evaluated further to determine its effects on a number of neurobehavioral and neurochemical endpoints, and these findings will be used to guide the selection of outcome measures that should be assessed in an ongoing epidemiological study of children whose mothers consumed PCB-contaminated Fox River fish. Because commercial Aroclor mixtures can differ between lot numbers, the comparison between our study and Roegge *et al.* (2004) gives indirect evidence for comparability of the toxicity of the PCB congener mixture with Aroclor 1254.

CONCLUSIONS

No individual formerly commercially available Aroclor provides an accurate representation of PCB profiles currently found in Fox River fish. Aroclor profile data is useful in calculating theoretical mixtures in different proportions designed to mimic a given PCB profile in an environmental exposure matrix. A mixture prepared with proportions of Aroclors 1242, 1248, 1254, and 1260 specified by the theoretical construct provided a dosing solution for laboratory studies which closely approximates actual environmental exposures in humans exposed to PCBs from Fox River fish. The dosing solution was found to have low dioxin-like TEQ activity and *in vivo* toxicity slightly greater than that of Aroclor 1254. We show that high activity toward enhancing microsomal RyR channel activity serves as a toxicologically relevant quantitative biomarker for complex environmental mixtures that lack AhR activity.

ACKNOWLEDGMENTS

This work was supported by grants ES11263 and ES11269 from NIEHS and # R-82939001 from U.S. EPA and the Hansen-Ducker Heritage Fund. The authors are grateful to Kanjana Imsilp for assisting with formulation of the mixtures and to Bob Paulson at the Wisconsin DNR for sharing data on the PCB congener profiles in Fox River fish.

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