Environmental Science & Technology

Article

pubs.acs.org/est

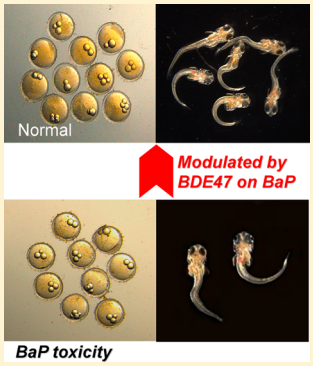
Modulation of Benzo[a]pyrene-Induced Toxic Effects in Japanese Medaka (*Oryzias latipes*) by 2,2′,4,4′-Tetrabromodiphenyl Ether

Yanbin Zhao, Kai Luo, Zhanlan Fan, Chong Huang, and Jianying Hu*

MOE Laboratory for Earth Surface Processes, College of Urban and Environmental Sciences, Peking University, Beijing 100871, China

Supporting Information

ABSTRACT: Because polycyclic aromatic hydrocarbons (PAHs) and polybrominated diphenylethers (PBDEs) are ubiquitous and coexist in the environment and in wildlife, there are potential interactions between them that could cause toxic effects. In this study, the modulating effects of 2,2′,4,4′-tetrabromodiphenyl ether (BDE47) and benzo[a]pyrene (BaP)-induced reproductive and developmental toxic effects in Japanese medaka (*Oryzias latipes*) were investigated by exposing adult Japanese medaka to BaP alone, BDE47 alone, and coexposing them with both BaP and BDE47 at different concentrations, respectively. Exposure to BaP alone significantly suppressed fecundity and egg protein content and markedly induced skeletal deformation in F1 generation eleutheroembryos. BDE47 significantly recovered reproductive functions, fecundity, and egg protein content, suppressed by BaP when the concentration of BDE47 increased to 0.44 μg/L. Such effects can be at least partly explained by the decreased BaP levels in the coexposure groups and the accompanying increase in the circulating level of 17β-estradiol in female medaka fish. The prevalence of skeletal deformations markedly increased to 19.3 ± 2.4% and 16.0 ± 1.6% in fish coexposed to BaP and BDE47 at 0.44 and 2.58 μg/L compared with that of fish exposed to 1.21 μg/L BaP alone (9.7 ± 1.7%), and the impacts on male medaka fish in the coexposure groups would be the crucial reason leading to these effects. Considering that the measured water concentrations of BaP and BDE47 in the present study were comparable with those reported in rivers and harbors, BaP and BDE47 contamination in the real world would have a significant level of interactive effects on wild fish.



INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) and polybrominated diphenyl ethers (PBDEs) are ubiquitous, persistent, bioaccumulative, and ecologically toxic environmental pollutants.[1−4] PAHs are generated primarily by incomplete combustion of organic matter in atmospheric emissions, and in some cases, petroleum-sourced PAHs are also a major contributor of pollution in surface and subsurface aquatic environments. PAHs with half-lives of 0.2−5 years in aquatic ecosystems[2,5] tend to deposit in sediment from which they are released and accumulate in various aquatic biota, e.g., macroinvertebrates, fish, and birds.[6,7] Additionally, PBDEs, which are an important group of brominated flame retardants, have emerged as contaminants with exponentially increasing environmental concentrations with doubling times of approximately 4−6 years.[8] Because of their great trophic magnification potential in the aquatic food web, PBDEs have been detected at high concentrations in wildlife at high trophic levels as well as in humans.[8,9] Thus, these two widespread contaminants often coexist at high levels in wildlife and humans.[10,11]

2,2′,4,4′-Tetrabromodiphenyl ether (BDE47) has been widely detected as a predominant PBDE congener contaminating wildlife and humans. Available toxicological evidence shows that BDE47 can disrupt thyroid hormone homeostasis, cause neurodevelopmental deficits, and exhibit immunotoxicity and reproductive toxicity in vertebrates.[12,13] One possible mechanism responsible for these impairments is that BDE47, as an exogenous ligand for the pregnane X receptor (PXR), can activate the PXR signaling pathway and subsequently up-regulate the transcription levels of cytochrome p4502B (*CYP2B*) and *CYP3A*, and enhance the elimination of endogenous hormones such as triiodothyronine (T3) and thyroxine (T4).[14] It has been acknowledged that PXR can regulate the metabolism of a great number of endogenous and exogenous ligands by regulating the gene expression of drug-metabolizing enzymes.[15] Recently, in vitro studies have also shown that PXR can modulate the metabolism of non PXR-ligands, such as PAHs, by regulating the transcription of genes that encode enzymes responsible for the metabolism of PAHs,[16] and also BDE47 could significantly reduce *CYP1A* expression, as demonstrated by a BaP-based cell exposure experiment.[17] Therefore, there is a potential that PBDEs may alter the metabolism of PAHs and modulate their toxicological effects in wildlife and humans. The reproductive toxic effects and skeletal deformations induced by PAHs in fish, birds, and mammals have been well documented;[1] however, no in vivo study has investigated the role of PBDEs in PAHs-induced toxic effects, even though these compounds are generally codetected in wildlife samples.

Of PAHs, benzo(a)pyrene (BaP) is an important environmental toxicant and is of great concern because of its potent

Received: January 8, 2013
Revised: October 23, 2013
Accepted: October 25, 2013
Published: October 25, 2013

carcinogenic and mutagenic properties and endocrine-disrupting activity.[18,19] To clarify the potential modulating effects of BDE47 on BaP toxic effects, adult Japanese medaka (*Oryzias latipes*) were exposed to BaP alone, BDE47 alone, and combinations of the two, and reproductive toxic effects and skeletal deformations induced by BaP were evaluated. Transcript abundances of genes involved in ovarian development and steroidogenesis, exogenous xenobiotic metabolism, and the levels of 17β-estradiol (E2) in fish after exposure and BaP-3sulfate (BaP-3Sul) in exposure water were also analyzed to better understand the effects of BDE47 exposure on the toxicity of BaP in Japanese medaka.

MATERIALS AND METHODS

Chemicals for Exposure. BaP (>95% purity) was purchased from Alfa Aesar (Tianjing, China), BDE47 (>98% purity) from ChemService (West Chester, PA, U.S.A.), and dimethyl sulfoxide (DMSO) from Sigma (St. Louis, MO, U.S.A.).

Animals. Adult Japanese medaka (stage 44), orange red strain, 14 weeks old (about 100 dph) with body weight of 470 mg and body length of 31 mm were selected from brood stock maintained for several years at our laboratory. These fish were cultured in 24 L flow-through tanks (about 24 pairs of fish per tank) under conditions that facilitated breeding (16:8 light/dark cycle) and were fed live brine shrimp (*Artemia nauplii*) twice daily. The tap water used in the experiment was filtered through activated carbon and had a hardness of 81.1 ± 1.2 mg/L $CaCO_3$, pH of 7.7 ± 0.2, dissolved oxygen of 7.8 ± 0.3 mg/L, and was maintained at 25 ± 1 °C.

Exposure. Prior to the initiation of exposure, 14 week old medaka fish were acclimated for two weeks in the experimental tanks (once-formed glass vessel with no seams) filled with 12 L of activated carbon-filtered tap water at a flow rate of 24 L/day using a flow-through design. Following the acclimation period, 12 pairs of medaka per glass tank were randomly assigned and exposed in a flow-through exposure (two tanks per treatment). DMSO solution of the chemical (200 μL) was added to 24 L activated carbon-filtered tap water in a stainless steel tank, and then the mixed exposure solution was slowly inflowed from the stock stainless steel tank into the exposure tanks at the flow rate of about 24L/d. To initially clarify BaP toxic effects, medaka were exposed to waterborne BaP with nominal concentrations of 0, 0.2, 1, 5, 25, and 125 μg/L (two tanks per treatment). To clarify the modulation effects of BDE47, medaka were exposed to waterborne BaP (25 μg/L), BDE47 (1, 10, and 100 μg/L), and their mixtures (BaP 25 μg/L + BDE47 1 μg/L; BaP 25 μg/L + BDE47 10 μg/L; and BaP 25 μg/L + BDE47 100 μg/L), together with a vehicle control (0.005% DMSO) (two tanks per treatment). Concentrations of chemicals in the water were measured by gas chromatography–mass spectrometry (GC-MS), and the details of the analytical processes are provided in the Supporting Information. Exposure was continued for five weeks, and no mortalities were observed. In the last exposure week, eggs from each treatment tank were collected for analysis of reproductive toxic effects, and the prevalence of eleutheroembryo skeletal deformities. On the last day of exposure, six males and six females (at about 150 dph) from the exposure tanks per treatment (three males and three females from each tank) were anaesthetized in ice cold water and sampled, and the gonad and liver were dissected from each fish and frozen in liquid nitrogen for gene expression analysis.

After the exposure experiments as described above, cross-fertilization experiments were carried out in clean water without addition of chemicals to clarify the mechanism of skeletal deformities. In the cross-fertilization experiments, females in the exposure group mated with males in the control group, or males in the exposure group mated with females in the control group. Eggs were collected on four consecutive days for observation of eleutheroembryo skeletal deformations.

Reproduction and Eleutheroembryo Development. Spawned eggs were collected daily at 2–3 h post-fertilization during the final exposure week. Mean numbers of spawned eggs per female were calculated for each exposure group. An intact spawned egg was homogenized in a glass tissue grinder, and then the total protein content was determined using a bicinchoninic acid (BCA) protein assay kit (Novagen, Rockford, IL), according to the manufacturer's instructions.

A Leika M165 FC microscope was used to observe eleutheroembryo development. Eleutheroembryos with skeletal deformations were identified based on the curvature of the spine (scoliosis) and writhing in the bottom of the tank. The prevalence of eleutheroembryos with skeletal deformation per day per treatment was calculated as the ratio of the number of skeletally deformed fry with any degree of spinal curvature to that of hatched fry.

Gene Expression. Total RNA was extracted from the liver and gonad samples using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocols. To prevent genomic DNA contamination, total RNA was digested by DNase I (TaKaRa Biotechnology, China) and then purified. First-strand cDNA synthesis and the relative quantitation in real time RT-PCR were performed according to methods described previously.[20] Gene expression was calculated as the fold-change relative to the mean expression of the solvent control group (fold induction = 1 for the solvent control). Primers for quantification of mRNA of each gene were designed using Premier Primer 5.0 (PREMIER biosoft international, U.S.A.), with all primer sequences shown in Table S1 of the Supporting Information. To minimize DNA contamination, most primer pairs were designed to span at least one intron of the genomic sequence. Ribosomal protein L7 (RPL-7) was used as the internal control,[20] and the variations of RPL-7 expression among samples for each group were below 12%, without significant difference among exposure groups. The mRNA quantification for each target gene was estimated as the value relative to RPL-7 by the comparative cycle threshold (Ct) method as described previously.[21]

Chemical Analysis. The concentrations of BDE47 and BaP in each exposure group were quantified once a week through the exposure period. Equal volumes (0.5 mL) of test solution collected from both chambers of each exposure group were combined, spiked with 10 ng perylene-d_{14}, and 10 ng PCB153 as internal standard for determining the concentration of BDE47 and BaP, and then subjected to liquid–liquid extraction three times. Each time the sample was mixed with 8 mL dichloromethane and then oscillated for 10 min. After adding sodium sulfate (Na_2SO_4) to remove moisture, the sample was dried using a gentle stream of nitrogen and reconstituted with 0.1 mL of n-hexane for GC-MS analysis. The fortification experiments were conducted by adding an amount of the surrogate standards comparable to the concentrations of BaP and/or BDE47 in water samples. The recoveries of perylene-d_{14} and PCB153 were 101.51 ± 2.44% and 97.59 ± 5.08%. The recoveries for spiked samples were 111 ± 8.2% for BaP and 102 ± 0.4% for BDE47. Method detection limits (MDLs) were 170 ng/L and 150 ng/L for BaP and BDE47, respectively. The limits of quantization (LOQ) of BaP and BDE47 were 550 ng/L and 500 ng/L,

respectively. The details for GC-MS analysis are provided in the Supporting Information.

The concentration of BaP-3 sulfate (BaP-3Sul) in the water and the concentration of E2 in the whole body of medaka fish (six females from the exposure tanks per concentration) were measured by ultra performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS). Details of the analytical processes are provided in the Supporting Information.

Statistical Analysis. Statistical analyses were conducted using SPSS 17.0 (SPSS, Chicago, IL, U.S.A.). Normality of each data set was assessed by the Shapiro−Wilk test, and the homogeneity of variance was determined by Levene's test. Differences among the exposure groups were analyzed by ANOVA followed by Tukey's test. Differences with $p < 0.05$ were considered significant.

RESULTS

Chemical Concentrations in the Water. The determined BaP and BDE47 concentrations in the water of each exposure group are shown in Table 1, which were much lower than the corresponding nominal concentrations and were environmentally relevant compared with those previously reported in aqueous environment.[22−24] The determined BaP concentrations in BaP-alone exposure groups were about 6.1−29.2 times lower than the corresponding nominal concentrations, and those in the four groups with coexposure to BDE47 were about 20.7−53.9 times lower than the nominal concentration of 25 μg/L BaP. Similarly, the determined concentrations of BDE47 in single BDE47 groups were about 13.0−25.6 times lower than the related nominal concentrations, and those in the coexposure groups were about 18.9−38.7 times lower than the corresponding nominal concentrations. The determined concentrations were below the detection limit for all vehicle control water.

Table 1. Concentrations of BaP and/or BDE47 in Water during the Exposure Period

BaP exposure groups		BaP + BDE47 exposure groups	
nominal concentration (μg/L)	measured concentration (μg/L)	nominal concentration (μg/L)	measured concentration (μg/L)
control	ND[a]	control	ND[a] for BDE47 and BaP
0.2	0.033 ± 0.012	1 (BDE47)	0.039 ± 0.019 (BDE47)
1	0.034 ± 0.020	10 (BDE47)	0.771 ± 0.170 (BDE47)
5	0.235 ± 0.063	100 (BDE47)	5.377 ± 1.604 (BDE47)
25	1.602 ± 0.148	25 (BaP)	1.206 ± 0.281 (BaP)
125	11.356 ± 0.144	25 (BaP); 1 (BDE47)	0.950 ± 0.118 (BaP); 0.053 ± 0.011 (BDE47)
		25 (BaP); 10 (BDE47)	0.564 ± 0.097 (BaP); 0.439 ± 0.124 (BDE47)
		25 (BaP); 100 (BDE47)	0.464 ± 0.056 (BaP); 2.584 ± 1.674 (BDE47)

[a]ND: not detected. Data are presented as means ± standard deviations ($n = 5$).

The concentration of BaP-3Sul in exposure aquaria was also measured. Though with limited information on its toxic effects, BaP-3Sul has been proved to be one of the predominant phase II metabolites of BaP[25] and can be more sensitively detected by the UPLC-MS-MS method compared with other major metabolites, such as 3-OHBaP or BaP-3-glucuronide. BaP-3Sul concentrations in BaP-alone exposure groups of 0.2, 1, 5, 25, and 125 μg/L were <0.0007, 0.003 ± 0.002, 0.057 ± 0.050, 0.323 ± 0.111, and 3.698 ± 1.159 ng/L, respectively, showing a dose-dependent increase. In BaP and BDE47 coexposure groups, BaP-3Sul concentrations were 0.506 ± 0.245, 1.025 ± 0.343, and 3.106 ± 1.606 ng/L at BDE47 nominal concentrations of 0, 1, and 10 μg/L, respectively. The concentration then dropped to 0.480 ± 0.132 ng/L at the BDE47 nominal concentration of 100 μg/L (Figure 1).

Reproductive and Developmental Toxic Effects of BaP and BDE47. During the fifth week of the BaP exposure period, the mean numbers of spawned eggs per female per day decreased significantly with increasing concentration of BaP from 0.034 to 11.356 μg/L (Figure 2A). The eggs spawned from the BaP exposure groups were colorless (Figure 3B), and the protein content in the 0.235, 1.602, and 11.356 μg/L BaP exposure groups was significantly lower than that of the control (Table S3, Supporting Information), indicating that degenerated eggs would be caused by BaP. Skeletal deformation was observed in F1 generation eleutheroembryos from the BaP exposure groups (Figure 3D), and the prevalence markedly increased with increasing BaP concentrations from 0.034 to 11.356 μg/L (Table 2).

Conversely, for fish exposed to BDE47 alone (0.04−5.28 μg/L), no noticeable effects upon the mean numbers or quality of spawned eggs or F1 skeletal deformation were observed compared to the control (Figures 2B and 4A and Table 2).

Alterations of BaP-Induced Toxic Effects with Coexposure to BDE47. The impacts on the mean numbers of spawned eggs per female per day for females exposed to 25 μg/L nominal concentrations of BaP were markedly reduced by BDE47. Results showed 12.3 ± 0.6 and 13.8 ± 1.2 eggs per female per day for females in coexposure groups when the concentrations of BDE47 were 0.44 and 2.58 μg/L, respectively, which were significantly higher than that (8.2 ± 0.9 eggs per female per day) in the 1.21 μg/L BaP exposure group ($p = 0.001$ and $p < 0.001$, respectively) (Figure 2B). Similar effects were also observed on protein content in the newly spawned eggs. While the protein content per egg was 63.1 ± 3.2 μg in fish exposed to 1.21 μg/L BaP, it increased significantly to 91.0 ± 3.7 μg and 97.1 ± 3.5 μg for fish coexposed to BaP and BDE47 at 0.44 and 2.58 μg/L, respectively ($p < 0.001$ and $p < 0.001$, respectively) (Figure 4A). Additionally, different from the reproductive toxic effects as described above, the developmental toxic effect, as exemplified by the prevalence of skeletal deformations for F1 generation eleutheroembryos in the coexposure groups, increased markedly to 19.3 ± 2.4% and 16.0 ± 1.6% at BDE47 concentrations of 0.44 and 2.58 μg/L, respectively, which was much higher than that (9.7 ± 1.7%) in the BaP alone (1.21 μg/L) exposure group (Table 2).

The vitellogenin (VTG-1) transcription level and E2 concentration in female medaka fish were further measured. The relative VTG-1 gene expression was significantly downregulated in females exposed to 1.21 μg/L BaP ($p = 0.022$). A dose-dependent increase in VTG-1 gene expression was observed in female fish coexposed to BaP and BDE47, with expression almost fully recovered even at 0.05 μg/L BDE47 (Figure 4B). Similarly, while E2 concentrations were 3.22 ± 0.30 ng/g dry weight in the whole bodies of control females, these significantly decreased to 0.83 ± 0.16 ng/g in females

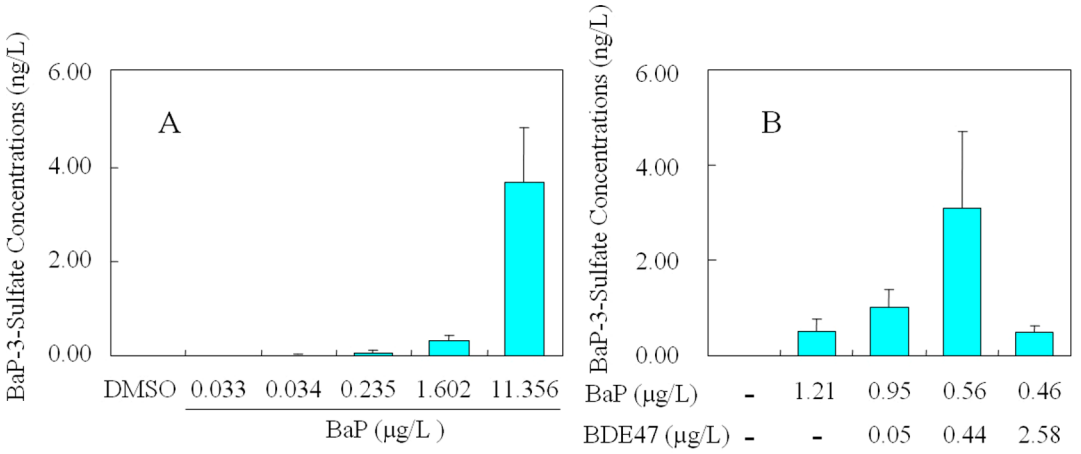


Figure 1. Variations of BaP-3-sulfate concentrations in the water of BaP-alone exposure groups (A) and in BaP and BDE47 coexposure groups (B). Data are presented as means ± standard deviations (n = 3).

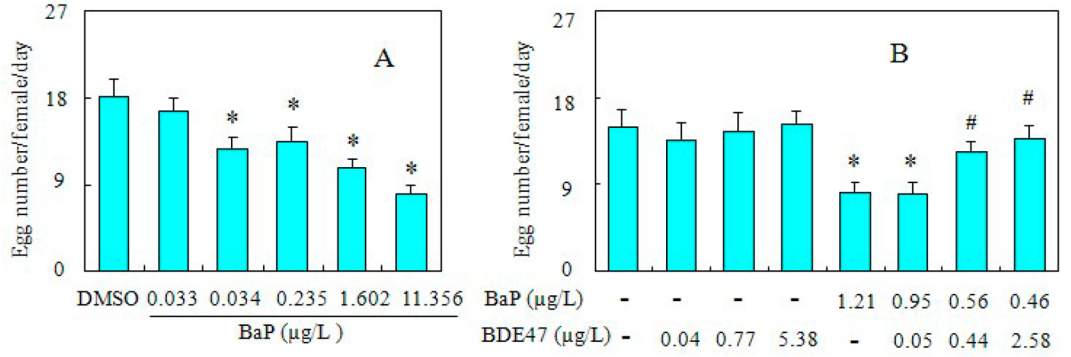


Figure 2. Mean numbers of spawned eggs per female per day during the last exposure week of medaka fish exposed to single BaP (A) and coexposed to BaP and BDE47 (B). Data are presented as means ± standard errors (n = 7). *: p < 0.05 compared with the control.

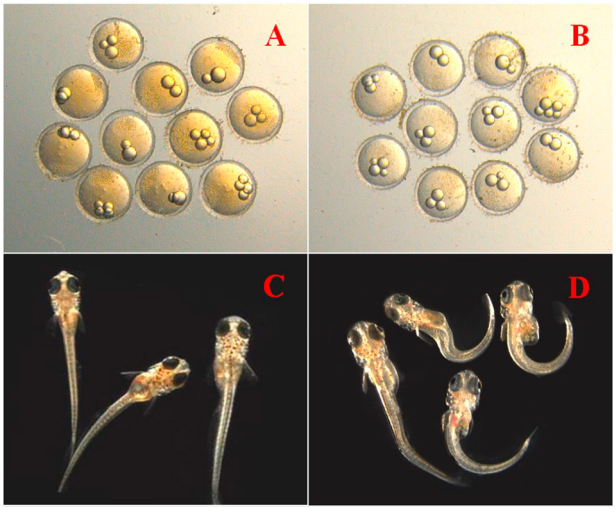


Figure 3. Degenerated eggs (as clarified by the colorlessness of eggs (about 10 hpf)) (B) and skeletal deformations in F1 eleutheroembryos (D) observed when adult medaka were exposed to BaP. (A) and (C) are the control.

exposed to 1.21 μg/L BaP (p = 0.003). Similar effects by BDE47 were observed in female fish exposed to BaP, and the E2 concentrations significantly recovered to 2.25 ± 0.17 ng/g and 3.07 ± 0.65 ng/g when the concentrations of BDE47 were 0.44 and 2.58 μg/L, respectively (Figure 4C).

Cross-fertilization experiments were also conducted after the exposure experiments to explore the potential reasons for the BDE47-mediated enhancement of BaP-induced F1 skeletal deformations. Prevalence of eleutheroembryo skeletal deformations were dose-dependently induced in groups in which male fish exposed to BaP were mated with female fish exposed to DMSO, while this phenomenon was not observed in the groups in which female fish exposed to BaP were mated with male fish exposed to DMSO (Table 2). Remarkable differences between sexes were found in groups exposed to BaP concentrations higher than 1.602 μg/L (Table 2). For groups in which male fish coexposed to BaP and BDE47 were mated with control females, the prevalence of eleutheroembryo skeletal deformations markedly increased to 11.7 ± 1.9% at 0.44 μg/L of BDE47 and 11.3 ± 2.5% at 2.58 μg/L of BDE47, respectively, which were higher than that (6.2 ± 1.6%) of the BaP-alone exposure group even at the same BaP nominal concentration of 25 μg/L. However, this phenomenon was not observed for the females (Table 2), indicating a male-specific modulation of BDE47 on the developmental toxicity of BaP.

Relative Gene Expression Levels. Transcription levels of genes involved in steroidogenesis and biotransformation in medaka gonads and phase I and phase II enzyme genes in medaka livers were further determined. Detailed data of all gene expressions are shown in Table S4 of the Supporting Information. Of six steroidogenic enzyme genes (*CYP11*, *CYP17*, *17β-HSD1*, *17β-HSD3*, *CYP19A*, and *20β-HSD*), *17β-HSD1* was significantly down-regulated by about 2-fold in both females and males

Table 2. Prevalence of Skeletal Deformation of Medaka Fish Exposed to BaP (n = 7) (A), Exposed to Single BDE47, or Coexposed to BaP and BDE47 (n = 7) (B) and in Cross-Fertilization Experiments after Parental Chemicals Exposure (n = 4) (C)[a]

BaP-alone exposure groups												
measured BaP concentration (μg/L)	ND[a]		0.033		0.034		0.235		1.602		11.356	
prevalence of skeletal deformation	0.02 ± 0.01		0.04 ± 0.01		0.10 ± 0.02		0.08 ± 0.02		0.12 ± 0.03		0.22 ± 0.03	
BaP and BDE47 coexposure groups												
measured BaP concentration (μg/L)	ND		ND		ND		ND		1.206		0.950	
measured BDE47 concentration (μg/L)	ND		0.039		0.771		5.377		ND		0.053	
prevalence of skeletal deformation	0.01 ± 0.01		0.01 ± 0.01		0.02 ± 0.01		0.02 ± 0.01		0.10 ± 0.02		0.09 ± 0.01	
Cross-fertilization experiments												
After BaP-alone exposure												
measured BaP concentration (μg/L)	0.033 (F)	0.034 (F)	0.235 (F)	1.602 (F)	11.356 (F)	0.033 (M)	0.034 (M)	0.235 (M)	1.602 (M)	11.356 (M)		
prevalence of skeletal deformation	0.02 ± 0.03	0.03 ± 0.03	0.03 ± 0.03	0.02 ± 0.00	0.03 ± 0.03	0.02 ± 0.03	0.02 ± 0.02	0.05 ± 0.03	0.09 ± 0.02	0.15 ± 0.07		
After coexposure to BaP (25 μg/L) and BDE47												
measured BaP concentration (μg/L)	1.206 (F)	0.950 (F)	0.564 (F)	0.464 (F)	1.206 (M)	0.950 (M)	0.564 (M)	0.464 (M)				
measured BDE47 concentration (μg/L)	ND	0.053	0.439	2.584	ND	0.053	0.439	2.584				
prevalence of skeletal deformation	0.02 ± 0.02	0.03 ± 0.03	0.02 ± 0.03	0.02 ± 0.03	0.06 ± 0.02	0.08 ± 0.03	0.12 ± 0.02	0.11 ± 0.03				

[a]ND: not detected. [a]F: Female fish after parental chemicals exposure. M: Male fish after parental chemicals exposure. Data are presented as means ± standard errors.

dx.doi.org/10.1021/es403260b | Environ. Sci. Technol. 2013, 47, 13068−13076

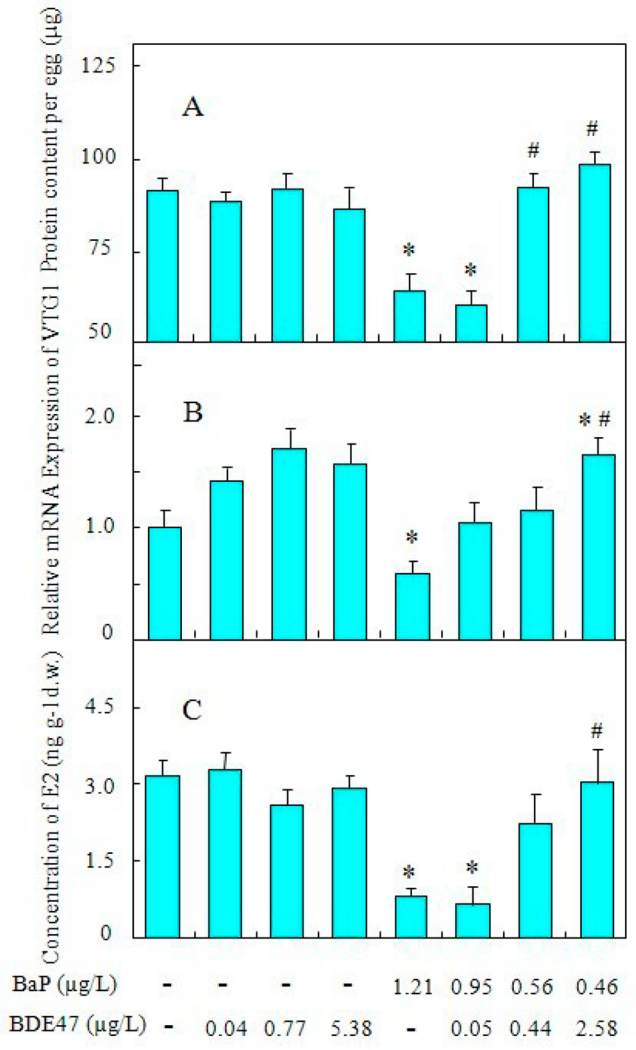


Figure 4. Variations in protein content per egg (A), relative mRNA expression of VTG1 (B), and concentrations of E2 in the whole body of females (C). RPL-7 was used as the internal control to calculate related mRNA expression. Data are presented as means ± standard errors (n = 10 for protein content per egg and n = 6 for mRNA expression of VTG1 and concentrations of E2). ∗: statistically significant differences between results of exposure group and results of the DMSO control. #: statistically significant differences between results in coexposure of BaP and BDE47 and results of the 1.21 μg/L BaP-alone exposure group.

exposed to 1.21 μg/L BaP compared with the control (p = 0.032 and p = 0.037, respectively), and dose-dependent up-regulation of *17β-HSD1* by BDE47 was observed in the BaP and BDE47 coexposure groups (Figure 5A and Table S4, Supporting Information). In the coexposure group, a statistically significant increase in *17β-HSD1* gene expression occurred at concentrations of 2.58 μg/L BDE47 relative to that of single BaP at 1.21 μg/L for both female and male medaka fish (p = 0.016 and p = 0.028, respectively). Of 13 phase I and phase II enzyme genes, sex-specific *CYP1A* and *CYP1B* expressions were observed (Figure 5 and Table S4, Supporting Information). *CYP1A* expression was significantly up-regulated (3.06 fold) in the livers of female medaka exposed to BaP alone of 1.21 μg/L (p = 0.017), showing relatively low expression. A dose-dependent decrease of *CYP1A* expression was observed in BaP and BDE47 coexposure groups. In males, *CYP1A* expression was slightly up-regulated (1.66 fold) in the BaP-alone exposure group at 1.21 μg/L but was significantly up-regulated (3.55 fold and 5.28 fold) in the coexposure groups at.0.44 and 2.58 μg/L of BDE47, respectively (p = 0.023 and p = 0.010, respectively). While no significant alterations of *CYP1B* were observed in female medaka fish, significant up-regulation (1.93 fold) was observed in the coexposure group at 2.58 μg/L of BDE47 (p = 0.041). It should be noted that although *CYP3A* slightly decreased in female medaka exposed to 1.21 μg/L BaP, significant up-regulations, 2.75 fold and 3.10 fold, were observed in the coexposure groups with concentrations of BDE47 were 0.44 and 2.58 μg/L, respectively. Similarly, the significant up-regulation (1.86 fold) of *CYP3A* was also observed in males for the concentration of BDE47 of 2.58 μg/L (Table S4, Supporting Information). Furthermore, the mRNA expression level of *p53*, an indicator of DNA damage, [26] was also measured in F_0 medaka gonads. Though there was no significant change in *p53* gene expression in the BaP-alone exposure group at 25 μg/L for both male and female fish, it was significantly up-regulated by BDE47 in males coexposed to BaP (1.64-fold and 2.36-fold) for the concentrations of BDE47 of 0.44 and 2.58 μg/L (p = 0.043 and p = 0.029, respectively). However this phenomenon was not observed for females in the coexposure groups (Figure 5D and Table S4, Supporting Information).

DISCUSSION

While increasing evidence shows that wildlife and human populations are being coexposed to multiple contaminants such as PAHs and PBDEs,[10,11] the potential interactions between them that cause toxic effects in fish have not been reported. The current study for the first time provides the evidence that PBDEs can modulate BaP-induced reproductive (decreased fecundity and degenerated eggs) and developmental toxic effects (skeletal deformations) in medaka fish.

The nominal concentrations of BaP and BDE47 were relatively high; however, their determined concentrations were 6.1−59.0 and 14.6−44.2 times lower than the nominal concentrations, respectively (Table 1), a phenomenon that has also been reported in previous papers.[27,28] In those papers, the measured concentrations were 0.34, 0.69, 12.19, and 25.70 μg/L, when Nile tilapia were exposed to BaP at the nominal concentrations of 10, 25, 50, 100 μg/L, respectively. The measured concentration of BDE47 was 0.23 ± 0.19 μg/L when Atlantic cod and turbot were exposed to BDE47 at the nominal concentration of 5 μg/L.[27,28] Such a phenomenon would be due to the fact that the partitioning to biota decreased the concentrations of BaP and BDE47 in water considering their high octanol−water partition coefficients (5.4 for BaP[29] and 6.81 for BDE47[30]). Dose characterization based on internal exposure that links bioavailability to effects should be further assessed as proposed by Escher and Hermnes.[31] It should be noted that the measured BaP concentrations were 0.46−0.95 μg/L in the coexposure groups, which are environmentally relevant in rivers and harbors as previously reported.[22,23] While the measured BDE47 concentrations (0.04−5.38 μg/L) were higher than those in rivers, a similar concentration (0.134 μg/L) has been observed in a wastewater sample.[24] In addition, the BaP concentration in water obviously decreased with increasing BDE47 concentration (Table 1). In this study, BDE47 markedly activated *CYP3A* (Table S4, Supporting Information), of which phenomenon has been also reported in previous paper and has been attributed to activation of the PXR signal pathway.[32] Because *CYP3A* is a major enzyme to catalyze BaP to 3-OHBaP, a main estrogenic metabolite,[33] such significantly up-regulated *CYP3A* under BDE47 exposure would modulate the metabolic processes of

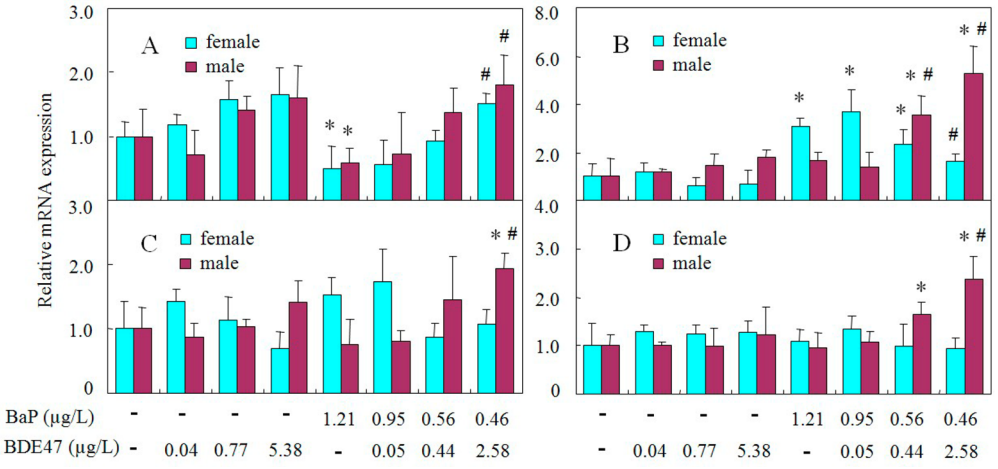


Figure 5. Relative mRNA expression of *17β-HSD1* (A), *CYP1A* (B), *CYP1B* (C), and *P53* (D). RPL-7 was used as the internal control to calculate related mRNA expression. Data are presented as means ± standard deviations. ∗: statistically significant differences between results of exposure group and results of the DMSO control. #: statistically significant differences between results in coexposure of BaP and BDE47 and results of the 1.21 μg/L single BaP exposure group.

BaP. The increasing trend of BaP-3Sul concentration with increasing concentration of BDE47 in BaP and BDE47 coexposure groups except for the highest BDE47 nominal concentration group (Figure 1) supports the above hypothesis. It should be noted that there was no reasonable explanation for the relatively low concentration of BaP-3Sul at 100 μg/L nominal concentration of BDE47, and further study of the metabolism pathway of BaP under coexistence with BDE47 is necessary.

Reduced fecundity and degenerated eggs, as exemplified by the colorlessness of eggs and the decline in egg protein, were observed in medaka fish exposed to BaP alone in the present study (Figures 2A, 3B, and 4A). The mechanism responsible for these toxic effects may relate to the ability of BaP to decrease circulating levels of E2 and therefore inhibit ovarian growth and spawning in female fish.[34,35] As shown in Figures 2B and 4A, however, the BaP-induced reproductive toxic effects (decreased fecundity and degenerated eggs) were reduced when medaka were coexposed to BaP and BDE47, which should be attributed to BDE47 modulation because no significant effects were elicited when fish were exposed to BDE47 alone (0.039−5.377 μg/L). A similar phenomenon was also observed for the transcription levels of vitellogenin in female medaka (Figure 4B). As an essential material for vitellogenesis and yolk biosynthesis in fish, VTG is a definite biomarker indicating oocyte maturation and egg quality.[36] Therefore, these effects of BDE47 on BaP-inhibited fecundity and degenerated eggs may be due to the fact that BDE47 recovered VTG gene expression in female fish exposed to BaP (Figure 4B). Considering that transcription of VTG is strongly dependent on the presence of estrogen in fish,[37] the recovered VTG was regarded as a consequence of E2 levels with similar alterations (Figure 4C). The determined BaP concentrations in water decreased with increasing BDE47 concentrations (Table 1), which would be responsible for the recovered E2 levels, and serves as a further reasonable explanation for the recovered reproductive activities. Additionally, the similar trend of *CYP1A*, an enzyme catalyzing the metabolism from E2 to 2-OHE2, with increasing BDE47 concentration supports the above hypothesis. It should be noted that *CYP1A* showed relatively low expression in the livers of medaka fish exposed to BaP, which is consistent with the previous studies, in which the gene expression of *CYP1A* induced by BaP in the liver was also limited to several fold, which was much lower than those in the gill, brain, and heart of vertebrates under subchronic BaP exposure.[38,39] The attenuation of BaP-induced *CYP1A* expression by BDE47 was also observed in a previous in vitro test, in which the pretreatment L02 cells with BDE47 could significantly decrease the *CYP1A* expression induced by BaP.[17] Another explanation was that the estrogenic metabolites of BaP, such as 3-OHBaP, could be markedly induced in the coexposure groups, which was supported by the mRNA expression trends of *CYP3A* and the increase of BaP-3-Sul, the phase II metabolite of 3-OHBaP, in culture water. On the other hand, BaP alone or in coexposure with BDE47 would also alter the biosynthesis of E2. In the present study, the dose-dependent up-regulation of *17β-HSD1* by BDE47 was observed in coexposure groups (Figure 5 and Table S4, Supporting Information). Since *17β-HSD1*, a steroidogenic enzyme, is involved in catalyzing the conversion of estrone (E1) to E2 in vertebrates,[40,41] the variation in *17β-HSD1* expression would also contribute to the recovered E2 level.

Exposure to PAHs during fish embryo development results in embryotoxicity and dysmorphogenesis, such as pericardial and yolk sac edema, cardiac arrhythmia, jaw reductions, and skeletal defects.[23,42] In the current study, we demonstrated that subchronic exposure of adult medaka fish to BaP caused similar skeletal deformities (scoliosis) in the F1 generation eleutheroembryos (Figure 3D). The spines of most skeletal deformational eleutheroembryos were curved from side to side, resembling a "C" or "S" rather than a straight line. These eleutheroembryos could not swim, always writhed in the bottom of the tank, and were dead within a week after hatching. The prevalence of skeletal deformations for F1 generation eleutheroembryo was markedly enhanced when the BaP concentration was up to 0.034 μg/L, and an obvious increase was observed from 0.034 to 11.356 μg/L (Table 2). In contrast with these reduced effects on reproductive toxic effects (decreased fecundity and degenerated eggs), BDE47 markedly enhanced the BaP-induced developmental toxic effect (skeletal deformations) in eleutheroembryos. In addition, the cross-fertilization experiments showed that skeletal deformations of eleutheroembryos were specifically induced when males in the BaP-alone exposure groups or in the BaP and BDE47 coexposure groups mated with females in the DMSO group (Table 2), demonstrating that male medaka fish were a crucial factor leading

to skeletal deformations. The previous studies indicated that skeletal developmental toxic effects caused by BaP were possibly due to the impairment of chondrogenesis and then inhibition of bone formation.[43,44] Such BaP teratogenicity was at least partially associated with genotoxicity induced by the carcinogenic metabolites of BaP.[23,43,44] It has been reported that *CYP1A* and *CYP1B* enzymes can catalyze BaP to form a recognized biologically active carcinogen, BaP-7, 8-dihydrodiol-9, 10-oxide (BPDE), which can form several kinds of adducts with DNA, subsequently leading to replication errors or mutations if not repaired in time.[45] In the present study, the transcription levels of *CYP1A* and *CYP1B* were significantly up-regulated by BDE47 in coexposure groups in male medaka fish but not in females, and the mRNA expressions of *p53*, an indicator of DNA damage,[26] were also dose-dependently up-regulated in testis but not in ovaries of medaka fish (Figure 5 and Table S4, Supporting Information). These results suggested that the toxic effects on sperm/spermatogenesis in adult male medaka fish would crucially lead to skeletal deformations of medaka eleutheroembryos, and further study on DNA damage using some methods such as Comet test in medaka gonads would be necessary in order to better understand the mechanism.

Overall, the present paper for the first time provides the evidence that BDE47 could significantly reduce the BaP-induced reproduction toxic effects and enhance BaP-induced skeletal deformations in Japanese medaka. These results will deepen the understanding of the potential health impacts of PAHs and PBDEs on both wildlife and humans. Furthermore, further investigations on the mechanisms responsible for the alternations of BaP by BDE47 are necessary. For example, both female and male fish were exposed in one tank for investigating the reproductive toxicity and observing larvae deformation; thus, the detection of metabolites in the culture water in the present study did not provide the chance to look at the differences between sexes in the metabolism of BaP. To well clarify the sex-specific toxicity, further study on sex-specific difference of BaP metabolism by analyzing broad metabolites is warranted.

■ ASSOCIATED CONTENT

Supporting Information

Detailed descriptions of methods for chemical and E2 analysis, gene primer sequences, and detailed data on the reproductive and developmental toxic effects and relative gene expression levels. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

* Tel: 86-10-62765520. Fax: 86-10-62765520. E-mail: hujy@urban.pku.edu.cn.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support from the National Basic Research Program of China [2012AA062802] and the National Natural Science Foundation of China [41330637 and 41171385] is gratefully acknowledged.

■ REFERENCES

(1) Peter, E. T. D. *PAHs: An Ecotoxicological Perspective*; Wiley: Chichester, U.K., 2003.

(2) Doick, K. J.; Klingelmann, E.; Burauel, P.; Jones, K. C.; Semple, K. T. Long-term fate of polychlorinated biphenyls and polycyclic aromatic hydrocarbons in an agricultural soil. *Environ. Sci. Technol.* **2005**, *39*, 3663−3670.

(3) Wang, Y.; Jiang, G.; Lam, P. K. S.; Li, A. Polybrominated diphenyl ether in the East Asian environment: A critical review. *Environ. Int.* **2007**, *33*, 963−973.

(4) de Wit, C. A. An overview of brominated flame retardants in the environment. *Chemosphere* **2002**, *46*, 583−624.

(5) Northcott, G. L.; Jones, K. C. Partitioning, extractability, and formation of nonextractable residues in soil. 1. Compound differences in aging and sequestration. *Environ. Sci. Technol.* **2001**, *35*, 1103−1110.

(6) Livingstone, D. R. The fate of organic xenobiotics in aquatic ecosystems: Quantitative and qualitative differences in biotransformation by invertebrates and fish. *Comp. Biochem. Physiol.* **1998**, *120*, 43−49.

(7) Kayal, S.; Connell, D. W. Polycyclic aromatic hydrocarbons in biota from the Brisbane River estuary, Australia. *Estuarine, Coastal Shelf Sci.* **1995**, *40*, 475−493.

(8) Hites, R. A. Polybrominated diphenyl ethers in the environment and in people: A meta-analysis of concentrations. *Environ. Sci. Technol.* **2004**, *38*, 945−956.

(9) Martin, M.; Lam, P. K. S.; Richardson, B. J. An Asian quandary: Where have all of the PBDEs gone? *Mar. Pollut. Bull.* **2004**, *49*, 375−382.

(10) Easton, M.; Luszniak, D.; Von der Geest, E. Preliminary examination of contaminant loadings in farmed salmon, wild salmon and commercial salmon feed. *Chemosphere* **2002**, *46* (7), 1053−1074.

(11) Ackerman, L. K.; Schwindt, A. R.; Simonich, S.; Koch, D. C.; Blett, T. F.; Schreck, C. B.; Kent, M. L.; Landers, D. H. Atmospherically deposited PBDEs, pesticides, PCBs, and PAHs in Western US National Park fish: Concentrations and consumption guidelines. *Environ. Sci. Technol.* **2008**, *42* (7), 2334−2341.

(12) Darnerud, P. O.; Eriksen, G. S.; Jóhannesson, T.; Larsen, P. B.; Viluksela, M. Polybrominated diphenyl ethers: Occurrence, dietary exposure, and toxicology. *Environ. Health Perspect.* **2001**, *109*, 49−68.

(13) Ye, R. R.; Lei, E. N.; Lam, M. H.; Chan, A. K.; Bo, J.; van de Merwe, J. P.; Fong, A. C.; Yang, M. M.; Lee, J. S.; Segner, H. E.; Wong, C. K.; Wu, R. S.; Au, D. W. Gender-specific modulation of immune system complement gene expression in marine medaka *Oryzias melastigma* following dietary exposure of BDE-47. *Environ. Sci. Pollut. Res.* **2012**, *19* (7), 2477−2487.

(14) Richardson, V. M.; Staskal, D. F.; Ross, D. G.; Diliberto, J. J.; DeVito, M. J.; Bimbaum, L. S. Possible mechanisms of thyroid hormone disruption in mice by BDE 47, a major polybrominated diphenyl ether congener. *Toxicol. Appl. Pharmacol.* **2008**, *226* (3), 244−250.

(15) Kliewer, S. A.; Willson, T. M. Regulation of xenobiotic and bile acid metabolism by the nuclear pregnane X receptor. *J. Lipid Res.* **2002**, *43* (3), 359−364.

(16) Naspinski, C.; Gu, X. S.; Zhou, G. D.; Mertens-Talcott, S. U.; Donnelly, K. C.; Tian, Y. A. Pregnane X receptor protects HepG2 cells from BaP-induced DNA damage. *Toxicol. Sci.* **2008**, *104* (1), 67−73.

(17) An, J.; Yin, L.; Shang, Y.; Zhong, Y.; Zhang, X.; Wu, M.; Yu, Z.; Sheng, G.; Fu, J.; Huang, Y. The combined effects of BDE47 and BaP on oxidatively generated DNA damage in L02 cells and the possible molecular mechanism. *Mutat. Res.* **2011**, *721* (2), 192−198.

(18) Phillips, D. H. Fifty years of benzo[a]pyrene. *Nature* **1983**, *303*, 468−472.

(19) Santodonato, J. Review of the estrogenic and antiestrogenic activity of polycyclic aromatic hydrocarbons: Relationship to carcinogenity. *Chemosphere* **1997**, *34* (4), 835−848.

(20) Zhang, Z. B.; Hu, J. Y. Development and validation of endogenous reference genes for expression profiling of medaka (*Oryzias latipes*) exposed to endocrine disrupting chemicals by quantitative real-time RT-PCR. *Toxicol. Sci.* **2007**, *95*, 356−368.

(21) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). *Methods* **2001**, *25* (4), 402−408.

(22) Zhang, L. F.; Dong, L.; Ren, L. J.; Shi, S. X.; Zhou, L.; Zhang, T.; Huang, Y. Concentration and source identification of polycyclic aromatic hydrocarbons and phthalic acid esters in the surface water of the Yangtze River Delta, China. *J. Environ. Sci.* **2012**, *24*, 335−342.
(23) Puffer, H.; Duncan, K.; Von Hofe, E.; Winkler, D.; Brewer, G.; Mondal, S. Benzo(a)pyrene: Studies of the Effects of This Ubiquitous Pollutant on Fishes. In *Oceans '79*; Institute of Electrical and Electronics Engineers, Inc.; New York, 1979; pp 398−400.
(24) Wang, J. X.; Jiang, D. Q.; Gu, Z. Y.; Yan, X. P. Multiwalled carbon nanotubes coated fibers for solid-phase microextraction of polybrominated diphenyl ethers in water and milk samples before gas chromatography with electron-capture detection. *J. Chromatogr. A* **2006**, *1137*, 8−14.
(25) Zaleski, J.; Bansal, S. K.; Gessner, T. Formation of glucuronide, sulphate and glutathione conjugates of benzo(a)pyrene metabolites in hepatocytes isolated from inbred strains of mice. *Carcinogenesis* **1983**, *4*, 1359−1366.
(26) Kastan, M. B.; Onyekwere, O.; Sidransky, D.; Vogelstein, B.; Craig, R. W. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.* **1991**, *51* (23), 6304−6311.
(27) Costa, J.; Ferreira, M.; Rey-Salgueiro, L.; Reis-Henriques, M. A. Comparison of the waterborne and dietary routes of exposure on the effects of benzo(a)pyrene on biotransformation pathways in Nile tilapia (*Oreochromis niloticus*). *Chemosphere* **2011**, *84*, 1452−1460.
(28) Sundt, R. C.; Pampanin, D. M.; Larsen, B. K.; Brede, C.; Herzke, D.; Bjornstad, A.; Andersen, O. K. The BEEP Stavanger workshop: Mesocosm exposures. *Aquat. Toxicol.* **2006**, *78S*, S5−S12.
(29) Dabestani, R. T.; Ivanov, I. N. A compilation of physical, spectroscopic, an photophysical properties of polycyclic aromatic hydrocarbons. *Photochem. Photobiol.* **1999**, *70*, 10−34.
(30) Braekevelt, E.; Tittlemier, S. A.; Tomy, G. T. Direct measurement of octanol−water partition coefficients of some environmentally relevant brominated diphenyl ether congeners. *Chemosphere* **2003**, *51*, 563−567.
(31) Escher, B. I.; Hermens, J. L. M. Internal exposure: Linking bioavailability to effects. *Environ. Sci. Technol.* **2004**, *38* (23), 455A−461A.
(32) Pacyniak, E. K.; Cheng, X.; Cunningham, M. L.; Crofton, K.; Klaassen, C. D.; Guo, G. L. The flame retardants, polybrominated diphenyl ethers, are pregnane X receptor activators. *Toxicol. Sci.* **2007**, *97* (1), 94−102.
(33) Bauer, E.; Guo, Z.; Ueng, Y.-F.; Bell, L. C.; Zeldin, D.; Guengerich, F. P. Oxidation of benzo[a]pyrene by recombinant human cytochrome P450 enzymes. *Chem. Res. Toxicol.* **1995**, *8*, 136−142.
(34) Johnson, L. L.; Stein, J. E.; Collier, T. K.; Casillas, E.; Varanasi, U. Indicators of reproductive development in prespawning female winter flounder (*Pleuronectes americanus*) from urban and non-urban estuaries in the northeast United States. *Sci. Total Environ.* **1994**, *141*, 241−260.
(35) Thomas, P.; Budiantara, L. Reproductive life history stages sensitive to oil and naphthalene in Atlantic croaker. *Mar. Environ. Res.* **1995**, *39* (1−4), 147−150.
(36) Matsubara, T.; Ohkubo, N.; Andoh, T.; Sullivan, C. V.; Hara, A. Two forms of vitellogenin, yielding two distinct lipovitellins, play different roles during oocyte maturation and early development of barfin flounder, *Verasper moseri*, a marine teleost that spawns pelagic eggs. *Dev. Biol.* **1999**, *213*, 18−32.
(37) Thomas, P. Reproductive endocrine function in female Atlantic croaker exposed to pollutants. *Mar. Environ. Res.* **1988**, *24*, 179−184.
(38) Wang, L.; Scheffler, B. E.; Willett, K. L. CYP1C1 messenger RNA expression is inducible by benzo[a]pyrene in *Fundulus heteroclitus* embryos and adults. *Toxicol. Sci.* **2006**, *93* (2), 331−340.
(39) Hoffmann, J. L.; Oris, J. T. Altered gene expression: A mechanism for reproductive toxicity in zebrafish exposed to benzo-[a]pyrene. *Aquat. Toxicol.* **2006**, *78* (4), 332−340.
(40) Scornaienchi, M. L.; Thornton, C.; Willett, K. L.; Wilson, J. Y. Cytochrome P450-mediated 17β-estradiol metabolism in zebrafish (*Danio rerio*). *J. Endocrinol.* **2010**, *206*, 317−325.
(41) Mindnich, R.; Deluca, D.; Adamski, J. Identification and characterization of 17β-hydroxysteroid dehydrogenases in the zebrafish *Danio rerio*. *Mol. Cell. Endocrinol.* **2004**, *215*, 19−30.
(42) Hose, J. E.; Hannaht, J. B.; Puffer, H. W.; Landolt, M. L. Histologic and skeletal abnormalities in benzo (a) pyrene-treated rainbow trout alevins. *Arch. Environ. Contam. Toxicol.* **1984**, *13* (6), 675−684.
(43) Kung, M. H.; Yukata, K.; O'Keefe, R. J.; Zuscik, M. J. Aryl hydrocarbon receptor-mediated impairment of chondrogenesis and fracture healing by cigarette smoke and benzo(a)pyrene. *J. Cell Physiol.* **2012**, *227*, 1062−1070.
(44) He, C. Y.; Zuo, Z. H.; Shi, X. A.; Li, R. X.; Chen, D. L.; Huang, X.; Chen, Y. X.; Wang, C. G. Effects of benzo(a)pyrene on the skeletal development of *Sebastiscus marmoratus* embryos and the molecular mechanism involved. *Aquat. Toxicol.* **2011**, *101*, 335−341.
(45) Uno, S.; Makishima, M. Benzo[a]pyrene toxicity and inflammatory disease. *Curr. Rheum. Rev.* **2009**, *5*, 266−271.