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Differential Toxicokinetics Determines the Sensitivity of Two Marine Embryonic Fish Exposed to Iranian Heavy Crude Oil

Jee-Hyun Jung,*[,†] Moonkoo Kim,[†] Un Hyuk Yim,[†] Sung Yong Ha,[†] Won Joon Shim,[†] Young Sun Chae,[†] Hana Kim,[†] John P. Incardona,[‡] Tiffany L. Linbo,[‡] and Jung-Hwan Kwon[§]

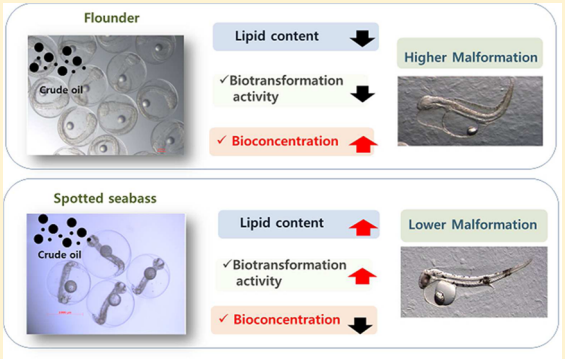
[†]Oil & POPs Research Group, Korea Institute of Ocean Science & Technology, 41 Jangmok1-gil Geoje, 53201, Korea

[‡]Environmental and Fisheries Science Division, Northwest Fisheries Science Center, National Marine Fisheries Service (NOAA), 2725 Montlake Boulevard East, Seattle, Washington 98112 United States

[§]Division of Environmental Science and Ecological Engineering, Korea University, 145 Anam-ro, Seongbuk-gu, Seoul 02841, Korea

Supporting Information

ABSTRACT: Interspecific difference in the developmental toxicity of crude oil to embryonic fish allows the prediction of injury extent to a number of resident fish species in oil spill sites. This study clarifies the comparative developmental effects of Iranian heavy crude oil (IHCO) on the differences of biouptake and toxic sensitivity between embryonic spotted sea bass (*Lateolabrax maculates*) and olive flounder (*Paralichthys olivaceus*). From 24 h after exposure to IHCO, several morphological defects were observed in both species of embryonic fish, including pericardial edema, dorsal curvature of the trunk, developmental delay, and reduced finfolds. The severity of defects was greater in flounder compared to that in sea bass. While flounder embryos accumulated higher embryo PAH concentrations than sea bass, the former showed significantly lower levels of CYP1A expression. Although bioconcentration ratios were similar between the two species for some PAHs, phenanthrenes and dibenzothiophenes showed selectively higher bioconcentration ratios in flounder, suggesting that this species has a reduced metabolic capacity for these compounds. While consistent with a conserved cardiotoxic mechanism for petrogenic PAHs across diverse marine and freshwater species, these findings indicate that species-specific differences in toxicokinetics can be an important factor underlying species' sensitivity to crude oil.



INTRODUCTION

Immediately after the *Hebei Spirit* oil spill (HSOS; December 7, 2007), many marine species were found dead on rocky shores and beaches, and more than 8571 ha of land-based fish aquaculture facilities were directly affected by the crude oil. Hatching success rate in marine fish species declined to lower than 50% in the vicinity of the spill, and there has been continuing controversy about the impacts of residual oil.[1,2] Surveys of juvenile fish populations revealed that some resident species, in particular the greenling (*Hexagrammos otakii*), showed low abundance rates up to four years following the HSOS.[3] This decrease was potentially due to an overlap of the timing of embryonic periods (from late November to December) of these species with the persistence of residual crude oil in near-shore areas. These findings prompted laboratory studies on the effects of crude oil from the HSOS on fish development.

The effects of crude oil on fish early life history stages were first highlighted after the 1989 *Exxon Valdez* oil spill contaminated nearshore spawning areas for Pacific herring (*Clupea pallasi*) and pink salmon (*Oncorhynchus gorbuscha*) with Alaska North Slope crude oil (ANSCO). Investigations carried out revealed that Pacific herring larvae hatching from adhesive demersal eggs at spawning sites affected by the spill had significantly higher frequencies of morphological deformities and cytogenetic abnormalities than those originating from unaffected sites.[4] In the ensuing decades, and further propelled by the 2010 *Deepwater Horizon* incident in the Gulf of Mexico, the effects of crude oils on developing fish have been extensively characterized at the cellular, organismal, and population level. Exposure of embryos of Gulf killifish (*Fundulus grandis*) and zebrafish (*Danio rerio*) to oiled sediments resulted in reduced hatching and developmental anomalies,[5,6] suggesting that oiled sediments can significantly impact early life stage in fishes.

The syndrome of developmental defects and mortality initially documented in field-collected herring and salmon larvae after the *Exxon Valdez* spill was linked to polycyclic aromatic hydrocarbons (PAHs), which make up an abundant

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fraction of most crude oils.[7−9] Subsequent research using the zebrafish model showed that the etiology of the syndrome was a disruption of embryonic cardiac function and morphogenesis.[10] This cardiotoxicity was specifically attributed to three-ringed PAHs,[11,12] and it is now established that crude oils from different geological sources, including the Iranian heavy crude oil (IHCO) spilled from the *Hebei Spirit,* disrupt heart development[12,13] in a diversity of fish species[14,15] by a mechanism involving the blockage of potassium and calcium ion channels essential for excitation−contraction (E−C) coupling in heart muscle cells.[16] It was further shown that externally normal embryos of zebrafish, Pacific herring, and pink salmon surviving trace crude oil exposures have permanently malformed hearts and reduced cardiorespiratory performance well into the first year of life,[17] potentially explaining the reductions in adult herring and pink salmon populations observed in both field and laboratory studies following the *Exxon Valdez* oil spill.[18−21]

These findings from the *Exxon Valdez* and *Deepwater Horizon* oil spills further warrant the investigation on the effects of the HSOS on fisheries and aquaculture resources and indicate the necessity for careful studies of the developmental cardiotoxicity of IHCO in indigenous species. In a previous study, we characterized the effects of the IHCO spilled from the *Hebei Spirit* in the zebrafish embryo model and compared its developmental toxicity syndrome to the well-studied ANSCO. Despite some differences in the physical and chemical properties of these two geologically distinct crude oils, the cardiotoxicity syndrome in developing zebrafish embryos caused by both oils was remarkably similar.[13] Olive flounder (*Paralichthys olivaceus*) and spotted sea bass (*Lateolabrax maculatus*) occur in the Pacific Ocean and Yellow Sea, especially off the coasts of Korea, Japan, and China. Both species are of commercial value, and olive flounder is the main species augmented by artificial seedling production and juvenile release.[22,23] Their breeding seasons are from late November to December, coinciding with the HSOS. They produce buoyant, pelagic eggs that develop in the upper water column, rendering them susceptible to exposure during the sea-surface oil-spill incident. Furthermore, there is no systematic comparative study on the toxicity under the environmentally relevant exposure system between fish species and weathering status. In this study, we evaluated ranges and interspecies differences in toxicity on fish embryos exposed to fresh and evaporated IHCO under environmentally relevant laboratory conditions. Bioconcentration of selected PAHs, malformation, and the level of CYP1A expression were also quantified for this purpose.

MATERIALS AND METHODS

Fish Embryos and Exposure. Eggs of olive flounder (*P. olivaceus*) and spotted sea bass (*L. maculates*) were artificially fertilized in a commercial fishery stations (IHWASANGROK and GEUNGYANG fishery station). At 6 h after fertilization, eggs were transported to the laboratory in Korea Institute of Ocean Science and Technology, Geoje, Korea. Eggs were acclimated for 24 h in flowing seawater at 16 °C on a 16 h light and 8 h dark photoperiod in the laboratory prior to the beginning of experiments. Floating eggs were collected and separated for the exposure experiments. Developing embryos (postfertilization: optic vesicle stage) were incubated in a 40 L exposure tank [40 cm (width) × 45 cm (length) × 24 cm (height)]. Experimental groups were control (no oil-treated gravel column), fresh IHCO (FIHCO), and weathered (or evaporated) IHCO (EIHCO)-treated gravel columns. EIHCO was prepared according to Jokuty et al.[24] by evaporating fresh oil to simulate the effect of volatilization, by which a considerable fraction of oil is removed during the initial weathering process immediately after a spill.[25] In short, 200 mL of fresh oil in a 10 L flask was placed in a rotary evaporator with a water bath filled with distilled water of 80 ± 5 °C. The flask was run at full speed (135 rpm) with an air flow of approximately 13 L/min through the flask. The flow rate was maintained by leaving the vacuum-release stopcock open to atmospheric pressure while a small vacuum pump was running. The oil was evaporated for 48 h to reach the almost constant total weight loss of approximately 30%. Gravel samples were coated with fresh or evaporated IHCO according to Jung et al.[13] A total of three grams of fresh or evaporated IHCO was loaded to 2 kg of gravel by manually shaking them together in an uncoated stainless steel container. Coated gravel was air-dried to a thin layer of soaked oil for 24 h and loaded into a generator column. Filtered water was continuously percolated (0.6 L h^{-1}) through the column to produce oil-contaminated seawater for toxicity studies. Water quality variables (i.e., pH, salinity, and dissolved oxygen) were monitored daily. The incubation temperature was maintained at 16 °C. Embryos were examined under a dissecting microscope, and abnormal or dead individuals were removed prior to the beginning of exposure. Embryos were exposed for 48 h to FIHCO and EIHCO gravel effluents. Toxicological end-points evaluated were pericardial edema, spinal curvature, tail fin defects, and developmental delay. In each experimental group, about 30 000 embryos were used in triplicate.

Imaging of Embryos with Confocal Microscopy. Developmental defects of embryos from oiled gravel effluent on the pericardial edema, tail fin defects, spinal curvature, and developmental delay were observed at 24 and 48 h after the onset of exposure. A total of 500 embryos from each triplicate were imaged and counted. At least ten embryos were randomly selected and mounted in 1.5% methylcellulose for viewing on the Zeiss Axioplan 2 compound microscope. Edema was quantified by measuring the pericardial area in left lateral images (collected on the stereoscope at 60× total magnification) using ImageJ (www.rsbweb.nih.gov/ij/) as described previously by Incardona et al.[26] Edema was measured as an increase in pericardial area by subtracting the average pericardial area measured in control embryos from all measures (controls and exposed) following conversion from pixels to μm^2 based on a stage micrometer image. A total of 30 embryos were fixed in 4% phosphate-buffered paraformaldehyde and processed for CYP1A and myosin heavy chain. Immunohistochemical staining was performed using rainbow trout CYP1A monoclonal antibody (Biosense) and myosin heavy chain cardiac, muscle staining (Biosense) for primary antibodies, and goat antimouse IgG_3 (g3) (Invitrogen) and goat anti-mouse IgG_{2b} (g2b labeled Alexa 488) (Invitrogen) for secondary antibodies. Staining intensity and occurrence of CYP1A were evaluated by fluorescence microscopy in each embryo. Immunolabeled embryos were mounted in 3% methylcellulose and imaged using a Zeiss LSM 780 Carl Zeiss Confocal system with Ar and HeNe lasers (Germany) (100× total magnification) as described by Jung et al.[13]

Quantitative Comparison of AhR2 and CYP1A mRNAs among Groups. Total RNA was extracted from embryos (30 mg wt) by Isogen (Wako). The purified total RNA was reverse-transcribed into cDNA using a first-strand cDNA synthesis kit

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Table 1. Oligonucleotide Primers and PCR Conditions Used in This Study

primer name	contains	sequence	PCR conditions
F CYP1A	probe	5′-/56-FAM/TTCACCATC/ZEN/CCACACT/31ABkFQ/-3′	10 s at 95 °C, 65 s at 59 °C, 60 s at 72 °C
	primer 1	5′-AAGTAGCCGTTCAGAGATGTG-3′	
	primer 2	5′-TTCGCCACTCTTCATTCCTG-3′	
Sb CYP1A	probe	5′ -/56-FAM/AAGCCGTTC/ZEN/CTGTATC/31ABkFQ/-3′	10 s at 95 °C, 65 s at 59 °C, 60 s at 72 °C
	primer 1	5′-AGGGTAAGTTGGGTTTGTCAG-3′	
	primer 2	5′-TGGTCTGTGATGTACTTGGTG-3′	
F AhR2	probe	5′-/56-FAM/AAGCCGTTC/ZEN/CTGTATC/31ABkFQ/-3′	10 s at 95 °C, 65 s at 60 °C, 60 s at 72 °C
	primer 1	5′-TAATCCTGGGCTACTCAGAGAC-3′	
	primer 2	5′-TCAGCGCAGTACATCATATC-3′	
Sb AhR 2	probe	5′-/56-FAM/TGAAAGGCT/ZEN/CCGGCTACCAATTCA/31ABkFQ/-3′	10 s at 95 °C, 65 s at 59 °C, 60 s at 72 °C
	primer 1	5′-AATGCCTGGGTACATGGTG-3′	
	primer 2	5′-TCAGCGCAGTGCATCATATC-3′	
F β-actin	probe	5′-56-FAM/TCATGAAGT/ZEN/GTGACGT/31ABkFQ/-3′	10 s at 95 °C, 65 s at 59 or 60 °C, 60 s at 72 °C
	primer 1	5′-AATGCCTGGGTACATGGTG-3′	
	primer 2	5′-CCTTGGAATGGAGTCTTGTGG-3′	
Sb β-actin	probe	5′-56-FAM/TATCCTGAC/ZEN/CCTGAAG/31ABkFQ/-3′	10 s at 95 °C, 65 s at 59 or 60 °C, 60 s at 72 °C
	primer 1	5′-TTCTCCATGTCATCCCAGTTG-3′	
	primer 2	5′-CAGAAGGACAGCTACGTTGG-3′	

Table 2. PAHs Concentrations (ng/L) in Oiled-Gravel Effluent of IHCO in the Study[a]

exposure hours	0 h			48 h		
coated oil type	control oiled-gravel effluent	FIHCO oiled-gravel effluent	EIHCO oiled-gravel effluent	control oiled-gravel effluent	FIHCO oiled-gravel effluent	EIHCO oiled-gravel effluent
Σ 16 PAHs (ng/L)	114	716	651	80	182	250
Σ Alkyl PAHs (ng/L)	146	10 600	9380	111	3890	5780
Σ PAHs (ng/L)	263	12 100	10 800	193	4340	6420
Σ Naph (ng/L)	180	6500	4960	126	1710	2630
Σ Flu (ng/L)	38	2090	2310	46	688	1270
Σ DBT (ng/L)	14	2230	2200	5	1410	1770
Σ Phen (ng/L)	25	1190	1240	12	520	732
Σ Chr (ng/L)	2.1	9.4	13	0.5	5.5	6.8

[a]Values represent means for duplicate water samples from the third exposure groups. Control, embryonic fish exposed to un-oiled gravel effluent; FIHCO, embryonic fish exposed to fresh IHCO gravel effluent; EIHCO, embryonic fish exposed to evaporated IHCO gravel effluent.

(Invitrogen). Quantitative PCR was performed using a two-step procedure. The β-actin gene was used as a positive sham for real-time PCR. PCR was performed using an initial denaturization step for 5 min at 95 °C, and then 40 cycles were run as follows: 30 s of denaturization at 95 °C, 30 s of annealing at 55 °C, and 1 min of extension at 72 °C. The ratio of optical density (OD) 260/280 was about 1.9, and the ratio of 260/230 was about 1.8 to 1.9. To quantify the mRNA expression level, we used the comparative CT methods ($2^{-\Delta\Delta CT}$ method) in Roto-Gene Q (Qiagen) according to the manufacturer's instruction. All experiments were performed in triplicate. The specific primers and probes for flounder AhR2 (fAhR 2), sea bass AhR2 (sbAhR2), flounder CYP1A (fCYP1A), and sea bass CYP1A (sbCYP1A) sequences were designed using the ABI PRISM Primer Express software (Applied Biosystems), and the details are shown in Table 1.

Water and Embryo Concentration of PAHs. *PAH Analyses.* The analyses were performed on two sample types: effluent water and fish embryo. Effluent water samples (approximately 2 L) were serially extracted three times with 50 mL of dichloromethane using a separatory funnel. Fish embryos were freeze-dried, and samples (0.2−1.0 g of dry weight) were extracted in a Soxhlet for 16 h with 200 mL of dichloromethane. Samples were spiked with surrogate standards (naphthalene-d_8, acenaphthene-d_{10}, dibenzothiophene-d_8, phenanthrene-d_{10}, chrysene-d_{12}, and perylene-d_{12}) before extraction. A 10 mL aliquot of the extract was used for lipid content determination with the gravimetric method. The remaining extracts were extensively cleaned using a deactivated silica−alumina column and HPLC with size-exclusion columns. The eluted samples were concentrated and taken up in *n*-hexane, and then terphenyl-d_{14} was added before the chemical analysis using a gas chromatograph (Hewlett−Packard HP6890) coupled with a quadrupole mass spectrometer (Hewlett−Packard HP5972). Quantification of the individual PAH components in the samples was performed using selected ion monitoring described in Yim et al.[27] A total of 16 priority PAHs by the U.S. Environmental Protection Agency (EPA) and selected alkyl-substituted PAHs were analyzed as follows: ($C_0 \sim C_3$) naphthalene, acenaphthylene, acenaphthene; ($C_0 \sim C_3$) fluorene; ($C_0 \sim C_3$) phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene; ($C_0 \sim C_3$) chrysene, benzo[*b*]-fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno-[1,2,3-*cd*]pyrene, dibenz[*a*,*h*]anthracene, benzo[*ghi*]perylene; and ($C_0 \sim C_3$) dibenzothiophene.

Quality Control and Quality Assurance. Surrogate standards were used for accounting the recovery of PAHs in each sample. Recoveries of target PAHs (16 priority PAHs) from the analyzed samples were 47−82%. For quality assurance of the fish embryo sample analysis, the certified reference materials

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Table 3. PAHs Concentrations (ng/g lipid weight) in Embryonic Sea Bass and Flounder at 24 and 48 h after Exposure to FIHCO and EIHCO[a]

exposure hours	24 h						48 h					
coated oil type	control	FIHCO	EIHCO	control	FIHCO	EIHCO	control	FIHCO	EIHCO	control	FIHCO	EIHCO
embryo species	sea bass	sea bass	sea bass	flounder	flounder	flounder	sea bass	sea bass	sea bass	flounder	flounder	flounder
Σ 16 PAHs (ng/g lipid weight)	7070	10 900	9720	20 600	30 900	69 300	1870	5240	7840	21 500	16 500	25 500
Σ alkyl PAHs (ng/g lipid weight)	16 900	48 200	54 200	41 200	32 0000	75 9000	13 500	77 400	88 100	60 376	25 0000	34 8000
Σ PAHs (ng/g lipid weight)	24 300	62 700	67 900	62 900	36 9000	88 3000	15 800	87 400	10 3000	83 194	28 0000	39 7000
Σ Naph (ng/g lipid weight)	15 600	25 700	27 000	35 700	13 2000	27 4000	36 00	35 100	38 600	34 200	10 0000	13 8000
Σ Flu (ng/g lipid weight)	119	4430	5100	nd	28 500	10 3000	1840	7100	7950	5640	19 400	30 000
Σ DBT (ng/g lipid weight)	4450	21 100	22 900	13 417	14 1000	35 1000	5060	29 200	36 400	19 800	11 1000	16 0000
Σ Phen (ng/g lipid weight)	3950	11 200	12 600	13 200	62 600	15 4000	4370	14 800	18 400	23 000	45 100	65 400
Σ Chr (ng/g lipid weight)	50	97	129	149	303	584	33	94	139	133	282	349

[a]Values represent means for pooled embryo samples from the third exposure groups. Control; PAHs concentration in embryonic fish exposed to un-oiled gravel effluent, FIHCO; PAHs concentration in embryonic fish exposed to fresh IHCO gravel effluent, EIHCO; PAHs concentration in embryonic fish exposed to evaporated IHCO gravel effluent.

(CRM) NIST 1947 provided by National Institute of Standards and Technology (Gaithersburg, MD, USA) were analyzed. PAH concentrations in the CRM were within the certified ranges. The PAH concentrations in the blank samples were not detected or lower than the detection limits of the method, which were 0.13 to 3.36 ng/g for biota and 0.09 to 5.67 ng/L for seawater. Relative percent differences of duplicate samples ranged from 0.19 (2-methylnaphthalene) to 19% (1-methyl-phenanthrene) for biota and from 0.13 (C4-fluorenes) to 21% (benzo[b]fluoranthene) for seawater.

Statistical Evaluation. All the data are expressed as mean ± s.d. In exposures involving treatment groups (e.g., control gravel and IHCO gravel), means were compared by a *t*-test. Differences between the control and exposed groups were analyzed using one-way ANOVA followed by Duncan's test. Differences showing $p < 0.05$ were considered as significant. The SPSS version 17.0 (SPSS Inc.) software package was used for statistical analysis.

RESULTS

PAH Concentrations in Oiled-Gravel Effluents and Embryos. Initial concentrations of total PAHs (∑PAHs) in oiled-gravel effluent (OGE) were 12 100 ng/L for FIHCO and 10 800 ng/L for EIHCO (Table 2), declining to 4340 and 6420 ng/L for FIHCO and EIHCO, respectively, after 48 h of column flow. This was 41 to 46 times higher in ∑PAH concentration than that in the effluent of the control clean gravel, which remained below 300 ng/L. The observed concentrations of individual PAHs also agreed well with those predicted using Raoult's law.[28] Relative compositions of PAH compounds were also similar for both FIHCO and EIHCO effluents (Table S1), with a dominance of naphthalenes (C0- to C4-) followed by dibenzothiophene, fluorine, and phenanthrene groups. However, the artificial weathering of EIHCO did result in lower levels of the more volatile naphthalenes and lower alkyl-substituted compounds, with a slight increase in less volatile, higher-molecular-weight compounds, such as C2- to C4-phenanthrenes. After 48 h, the column-flow concentrations of C0- to C2-naphthalenes were decreased in both FIHCO and EIHCO to a much higher degree than those of the more hydrophobic alkyl-phenanthrenes and alkyl-dibenzothiophenes (Table S1).

Flounder embryos accumulated higher levels of PAHs than did sea bass, and this species also differed in the changes of embryo PAH concentrations over time (Table 3). At 24 h of exposure, sea bass embryos accumulated ∑PAH of 62 700 ng/g of lipid and 67 900 ng/g of lipid from FIHCO and EIHCO OGEs, respectively, with embryo concentrations increasing to 87 400 ng/g of lipid and 103 000 of ng/g lipid, respectively, at 48 h. ∑PAH concentrations in control sea bass embryos were only 15 800 ng/g of lipid. In contrast, embryo PAH levels peaked in flounder embryos at 24 h, with ∑PAH 369 000 ng/g of lipid and 883 000 ng/g of lipid from FIHCO and EIHCO OGEs, respectively, dropping (but remaining higher than sea bass) at the 48 h time point to 280 000 ng/g of lipid and 397 000 ng/g of lipid, respectively. ∑PAH levels were 83 200 ng/g of lipid in flounder embryos exposed to control effluent. Embryos exposed to control effluents exhibited levels only 3 to 14 times lower for ∑PAH than did the embryos exposed to oiled-gravel effluent, while ∑PAH in the oiled effluent water was 41 to 46 times higher than in the effluent from control clean gravel. This relatively small difference in PAH concentration between oiled and control embryos is mainly due to elevated levels in the control embryos. The high levels in control embryos are most likely due to maternal transfer of PAHs from adult fish to embryo. It also should be noted that the PAH level in flounder embryos after 24 h exposure to EIHCO is highly elevated when compared with that in those exposed to FIHCO, while the concentration difference is not evident in the exposure waters. Due to a dynamic condition in the exposure system, embryos that are floating on the water surface can be contaminated by oil slicks or droplets that are not completely removed during the initial wash-out of oil-coated gravels prior to the exposure. In spite of the discrepancy, all presented PAH concentration data exhibit a consistent trend.

The most dominant PAHs in the both species were dibenzothiophene homologues (4450−351 000 ng/g of lipid)

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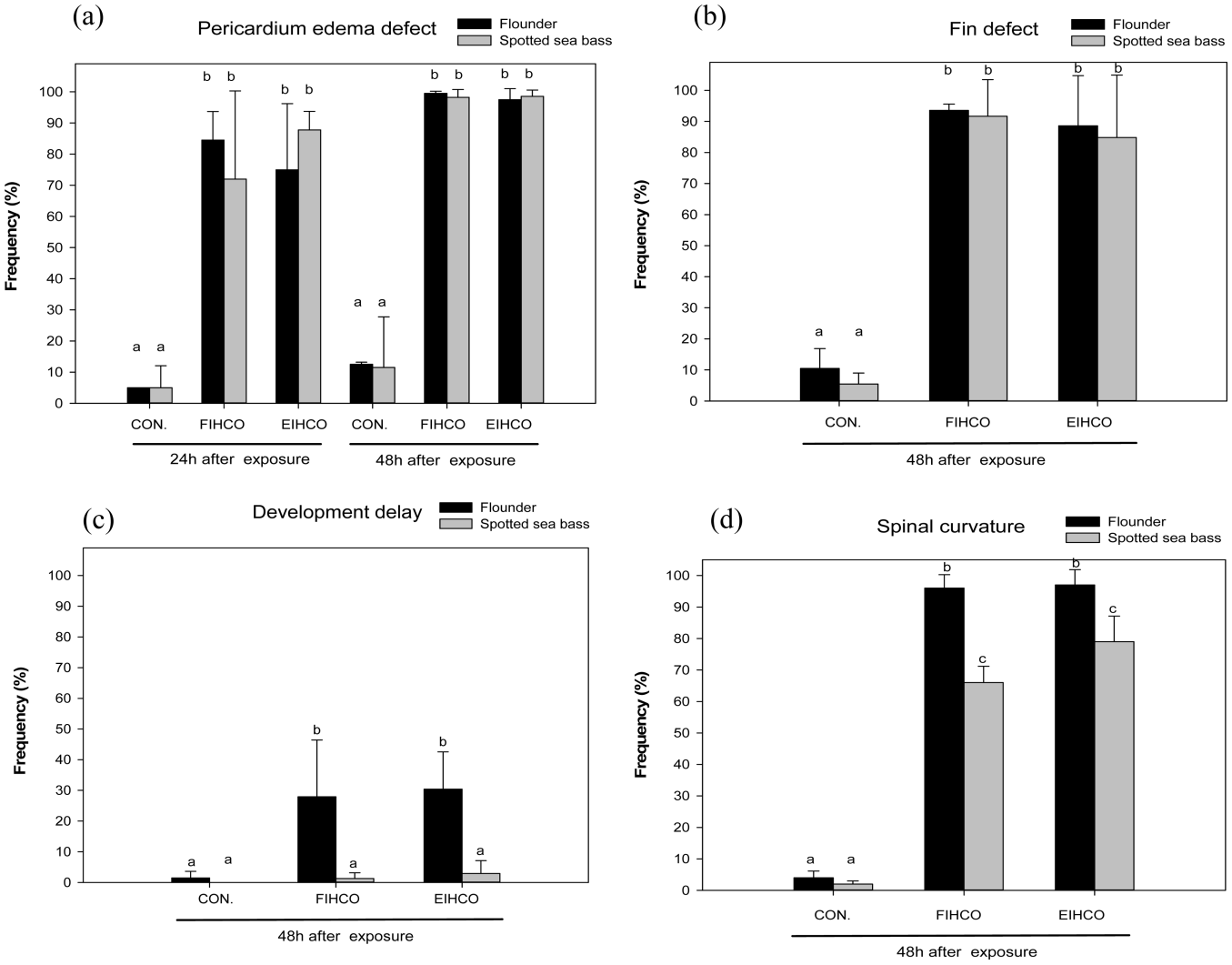

Figure 1. Gross morphology of embryonic olive flounder (*Paralichthys olivaceus*) and spotted sea bass (*Lateolabrax maculates*) at 24 and 48 h after exposure to FIHCO and EIHCO. Control, embryonic fish exposed to unoiled gravel effluent; FIHCO, embryonic fish exposed to fresh IHCO gravel effluent; EIHCO, embryonic fish exposed to evaporated IHCO gravel effluent. (a) Frequency of pericardial edema. (b) Frequency of fin defect. (c) Frequency of developmental delay. (d) Frequency of spinal curvature. Data are mean ± s.d. of three replicates (300 embryos). Data were subjected to ANOVA followed by Duncan's multiple-range test. Different letters denote statistically significant results ($p < 0.05$).

followed by the naphthalene group (3600−274 000 ng/g of lipid) (Table S2). Examination of individual embryo PAH concentrations (Table S2) revealed marked differences in the concentration ratios, calculated as the ratio of embryo PAH to water PAH, both between sea bass and flounder and among individual PAHs within each species (Table S3). In sea bass, most of the embryo-to-water-concentration ratios were below 20 and followed an expected trend of increasing ratios with increasing hydrophobicity (increasing molecular weight and degree of alkylation). In contrast, the concentration ratios for flounder were both markedly higher and did not follow a simple pattern associated with hydrophobicity, e.g., above 100 for phenanthrenes and as high as 254 for 2/3-methyldibenzothiophene but more similar to that of sea bass for naphthalenes and fluorenes (Table S3).

Morphological Abnormalities from Crude Oil Exposure. A suite of defects, including pericardial edema, reduced finfolds, dorsal body axis curvature and developmental delay were observed in the embryos of both species exposed to FIHCO and EIHCO gravel effluent (Figure 1). By some measures, flounder embryos were more severely affected. At 48 h after exposure, pericardial edema was observed at very high frequencies: 99.5 ± 0.7 and 97.5 ± 3.5% in flounder (FIHCO and EIHCO, respectively) and 98.2 ± 2.4 and 98.5 ± 2.0% in spotted sea bass (FIHCO and EIHCO, respectively) (Figure 1a). Frequencies of reduced finfold growth were similarly high in both species, at 93.5 ± 2.0 and 88.6 ± 16.1% (FIHCO and EIHCO, respectively) in flounder and 91.6 ± 11.8 and 84.8 ± 20.0 in embryonic sea bass (FIHCO and EIHCO, respectively) (Figure 1b). There were no statistically significant differences between embryonic flounder and sea bass in either exposure group. Developmental delay, indicated by reduced dorsal rotation of the head, immature pectoral fin buds, and pigment pattern, was statistically significant more frequently in embryonic flounder than in spotted sea bass, occurring in 27.9 ± 18.4% and 30.4 ± 12.1% of FIHCO and EIHCO exposure groups in flounder, respectively (Figure 1c). Dorsal body axis curvature was also observed more frequently in embryonic flounder, occurring in 96 ± 10.3% and 97 ± 8.9 of FIHCO and EIHCO exposure groups, respectively, compared to 66 ± 5.2% and 79 ± 8.1% in sea bass (Figure 1d). There

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were statistically significant differences between embryonic flounder and sea bass in either exposure group ($p < 0.05$).

Although the frequency of pericardial edema was similar in both species, flounder showed more severe edema in individual embryos. The pericardial area was significantly increased relative to controls in all oil-exposed groups ($p < 0.001$), but within each species, there was no significant difference in the severity of edema between the different oil-exposed groups (FIHCO versus EIHCO). However, the edema area in embryonic flounder (46.3 ± 11.9 μm^2 and 49.4 ± 17.8 μm^2 in embryos exposed to FIHCO and EIHCO OGE, control pericardial areas of 9.90 ± 1.4 μm^2 of control pericardial areas) was significantly higher than spotted sea bass (22.6 ± 2.3 μm and 28.6 ± 5.1 μm, respectively) (Figure 2).

Expression of the Biotransformation System. The AhR2 mRNA in oil-exposed groups was significantly higher than those of the control. The expression level of AhR2 mRNA in spotted sea bass was higher than that of flounder, and there was a significant difference between FIHCO and EIHCO groups after 24 h of exposure. In embryonic flounder, there was

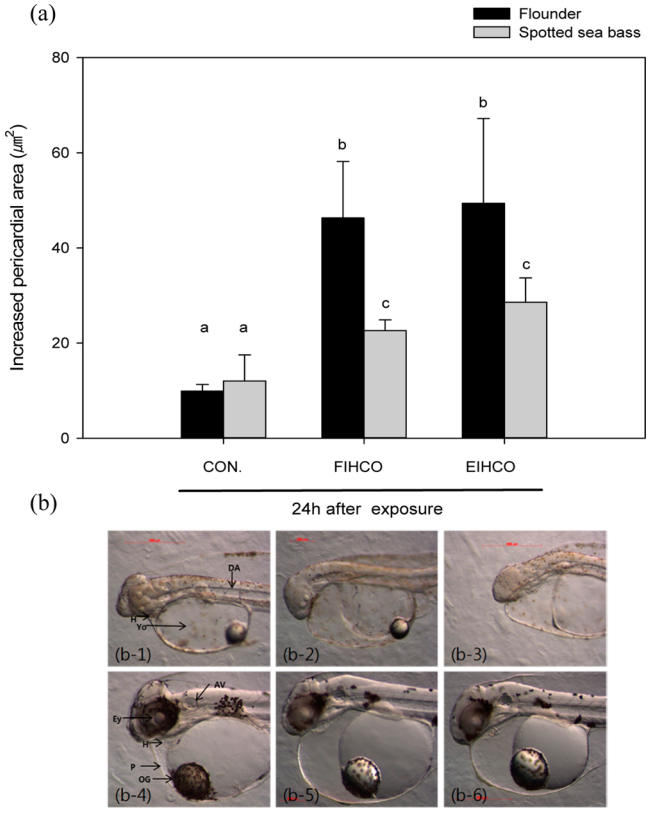

Figure 2. Comparative cardiotoxicity in embryonic olive flounder (*P. olivaceus*) and spotted sea bass (*L. maculates*) at 24 and 48 h after exposure to FIHCO and EIHCO. Incidence of edema was scored by measuring each video clip. (a) The area of edema compared to the embryonic fish exposed to unoiled gravel effluent. (b) Gross morphology of embryonic olive flounder and spotted sea bass at 24 and 48 h after exposure to FIHCO and EIHCO. (b-1) Control olive flounder, (b-2) olive flounder exposed to FIHCO, (b-3) olive flounder exposed to EIHCO, (b-4) control spotted sea bass, (b-5) spotted sea bass exposed to FIHCO (b-6), spotted sea bass exposed to EIHCO. H: heart, Yo: yolk, DA: dorsal aorta, Ey: eye, AV: auditory vesicle, PC: pericardium, OG: oil globule. Data are means ± s.d. Data were subjected to ANOVA followed by Duncan's multiple-range test. Different letters denote statistically significant results ($p < 0.05$).

no statistically significant difference in AhR2 mRNA expression between 24 and 48 h after exposure to FIHCO and EIHCO (Figure 3a). The expression level of CYP1A mRNA was

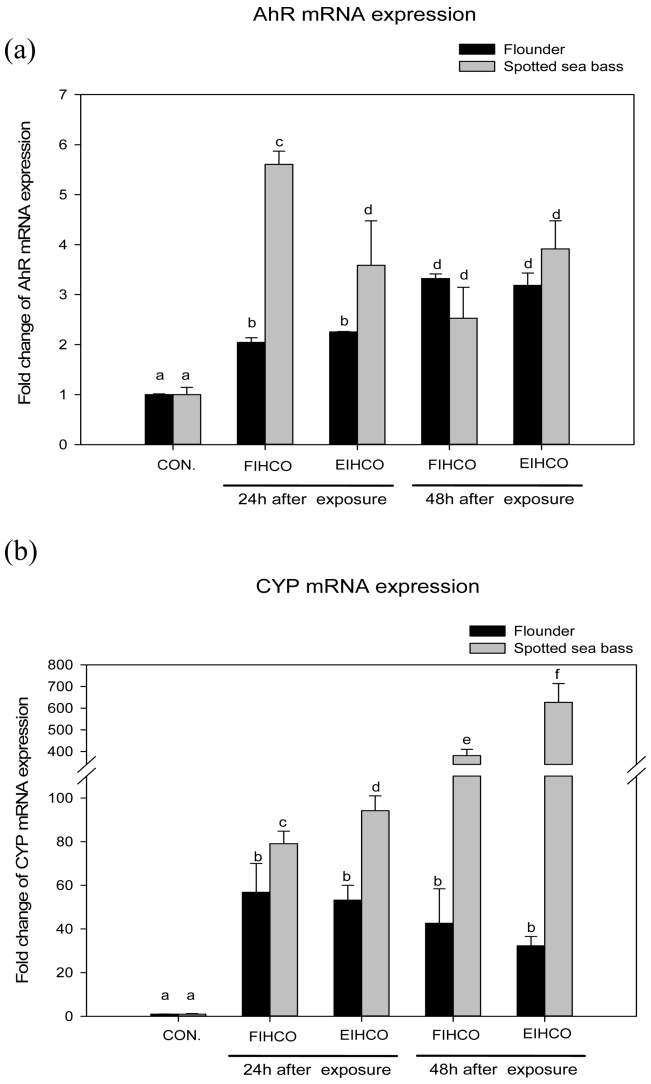

Figure 3. Level of relative mRNA expressions in embryonic olive flounder and spotted sea bass at 24 and 48 h after exposure to FIHCO and EIHCO. (a) Fold-change of the AhR2 mRNA. (b) Fold-change of the CYP1A mRNA. Data are means ± s.d. Data were subjected to ANOVA followed by Duncan's multiple-range test. Different letters denote statistically significant results ($p < 0.05$).

increased in all of exposure groups. The level of CYP1A mRNA in spotted sea bass was higher than those of flounder at 24 and 48 h after exposure and was the highest in embryonic spotted sea bass exposed to EIHCO (Figure 3b). Figure 4a shows significant induction of CYP1A protein in spotted sea bass exposed to FIHCO and EIHCO. The CYP1A protein expression level in spotted sea bass was higher than those of embryonic flounder exposure to FIHCO and EIHCO. Intense CYP1A immunofluorescence was induced strongly in the epidermis of the head, urinary pores, and eyes of embryos exposed to FIHCO and EIHCO gravel effluent (Figure 4).

Physical Properties and Lipid Contents in Embryos. Flounder embryos were smaller (1.00 mm in diameter, 0.36 mg in weight) than those of spotted sea bass (1.35 mm in diameter, 1.5 mg in weight), and the chorion was thinner as observed

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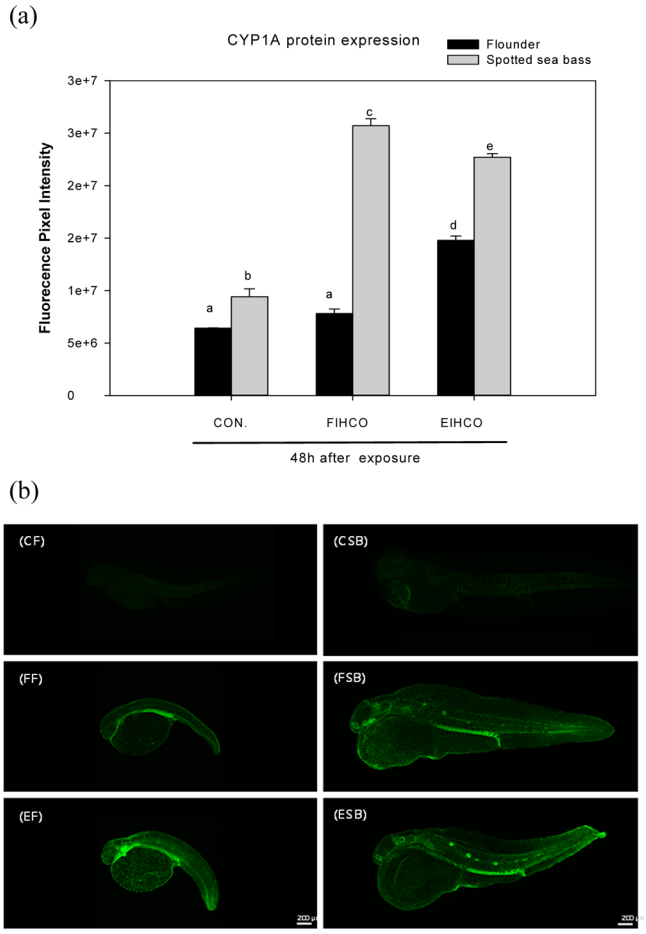

Figure 4. Level of relative CYP1A protein expressions in embryonic olive flounder and spotted sea bass at 24 and 48 h after exposure to FIHCO and EIHCO. (a) Intensity of CYP1A immunofluorescence. (b) Immunofluorecent activity in olive flounder and spotted sea bass at 24 and 48 h after exposure. CF, olive flounder exposed to unoiled gravel effluent; FF, olive flounder exposed to fresh IHCO gravel effluent; EF, olive flounder exposed to evaporated IHCO gravel effluent; CSB, spotted sea bass exposed to unoiled gravel effluent; FSB, spotted sea bass exposed to fresh IHCO gravel effluent; ESB, spotted sea bass exposed to evaporated IHCO gravel effluent. Data are means ± s.d. Data were subjected to ANOVA followed by Duncan's multiple-range test. Different letters denote statistically significant results ($p < 0.05$).

under microscopy (flounder, 0.18 μm; sea bass, 0.23 μm). The calculated surface-to-volume ratios for flounder and sea bass embryos were 6.04 and 4.44 mm^{-1}, respectively. Lipid contents measured in flounder and sea bass embryos were 0.05 and 0.22 g of lipid per g of dry weight, respectively.

DISCUSSION

The findings here provide further support for a highly conserved cardiotoxic response of teleost embryos following exposure to a several geologically distinct crude oils. Our results with olive flounder and spotted sea bass demonstrate a suite of defects from IHCO exposure in temperate perciform species that is nearly identical to that observed with ANSCO exposure in other evolutionarily older cold-water species (e.g., clupeiform herring and salmonids[14,18]) and with Louisiana MC252 crude-oil exposure in tropical perciforms (e.g., yellowfin tuna[15]). However, this study also highlights important species-specific differences in relative sensitivities to the same crude oil exposure. Although we did not perform dose–response tests to determine toxicity thresholds in both species, flounder accumulated more PAHs than did sea bass on the lipid weight basis and showed lower xenobiotic defense responses. Dorsal curvature and pericardial area were also observed to be more severely affected than those factors in sea bass in response to the same exposure concentrations.

Consistent with our previous observation in zebrafish,[13] in the current study, three dominant malformations were observed in both species, including pericardial edema, reduced finfold outgrowth, and dorsal curvature of the trunk and tail. Generally, all defects were more severe in flounder embryos, which also showed a much greater degree of developmental delay. Consistent with the severity of defects, flounder embryos accumulated higher embryo levels of PAHs but paradoxically showed a lower level of CYP1A induction measured by both mRNA and protein. This agrees well with the lower expression level of AhR receptors and CYP1A in flounder. CYP1A expression has a major role in protection from toxic effects by excretion xenobiotics, including PAHs. Higher biotransformation activity (CYP1A expression levels) in embryonic sea bass means greater elimination from body residue that leads the low lipid-normalized residue concentration of PAHs in embryos. Petersen and Kristensen[29] have reported that zebrafish do not metabolize some individual PAHs in the larval stage. However, our finding provides the evidence that there is a strong negative correlation between the lipid normalized concentration ratios and AhR-mediated biotransformation responses in embryonic marine fish. The data presented here thus provide insight into the relationships between oil weathering, PAH bioaccumulation, malformations, and the AhR and CYP1A pathway and how these relationships might differ between the two species tested.

The relationship between the severity of toxic effects and the weathering state was first characterized with ANSCO[7,8] and has been established for MC252 crude oil as well.[12,15] Artificial weathering of IHCO led to a depletion of naphthalenes and some relative enrichment of the tricyclic PAH families, but there were not significant differences for malformation rates between fresh and evaporated IHCO for either species. However, this is most likely due to the fact that the concentrations tested were already at the high end of the dose–response relationship (e.g., nearly 100% of animals had edema). In contrast, there were significant differences in CYP1A induction from fresh versus evaporated IHCO, consistent with an enrichment of more potent AhR ligands in the latter that typically are associated with higher-molecular-weight PAH fractions.[30]

The manifestation of developmental toxicity from crude oil is related to both toxicokinetic and toxicodynamic processes. In general, the suite of defects we observed here in olive flounder and spotted sea bass occurred at PAH concentrations producing toxicity in other fish species previously studied, i.e., $\leq$ 10 μg/L $\sum$PAH. Nearly 100% of flounder and sea bass embryos showed pericardial edema at that concentration, and on the basis of comparisons to other tropical and temperate perciforms,[12] thresholds for cardiotoxicity for these species are likely at least an order of magnitude lower. Thus, the molecular targets of crude oil involved in E–C coupling are likely the same in most, if not all, perciform species. Unlike previous studies, however, the simultaneous exposure of these two species to a common oiled-gravel effluent revealed interspecies

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Table 4. Logarithm of the Concentration Ratios ($C_{embryo}/C_{seawater}$ in L/kg_{lipid}) at 24 and 48 h after Exposure to Fresh (FIHCO) and Evaporated IHCO (EIHCO) for Individual Polycyclic Aromatic Hydrocarbons Analyzed[a]

	exposure duration	24 h	24 h	24 h	24 h	48 h	48 h	48 h	48 h
	coated oil type	FIHCO	EIHCO	FIHCO	EIHCO	FIHCO	EIHCO	FIHCO	EIHCO
	species	sea bass	sea bass	flounder	flounder	sea bass	sea bass	flounder	flounder
EPA priority 16 PAHs	naphthalene	4.72	5.00	5.05	5.68	4.63	5.08	5.20	5.59
	acenaphthylene	–	–	–	–	–	–	–	–
	acenaphthene	–	–	–	–	–	–	–	–
	fluorene	3.19	3.26	3.88	4.34	4.12	4.05	4.46	4.48
	phenanthrene	3.91	3.93	4.58	5.01	4.46	4.42	4.94	4.96
	anthracene	3.77	3.66	4.32	4.70	4.19	4.10	4.75	4.83
	fluoranthene	4.61	4.59	5.12	5.52	4.82	4.81	5.20	5.27
	pyrene	4.44	4.40	5.00	5.36	4.63	4.65	5.11	5.05
	benz[*a*]anthracene	–	–	–	–	–	–	–	–
	chrysene	4.43	4.49	4.92	5.15	4.55	4.62	5.03	5.02
	benzo[*b*]fluoranthene	–	–	–	–	–	–	–	–
	benzo[*k*]fluoranthene	–	–	–	–	–	–	–	–
	benzo[*a*]pyrene	–	–	–	–	–	–	–	–
	indeno[1,2,3-*c*,*d*]pyrene	–	–	–	–	–	–	–	–
	dibenz[*a*,*h*]anthracene	–	–	–	–	–	–	–	–
	benzo[*g*,*h*,*i*]perylene	–	–	–	–	–	–	–	–
other PAHs	dibenzothiophene	3.68	3.75	4.39	4.88	4.26	4.24	4.70	4.78
alkylated PAHs	1-methylnaphthalene	2.39	2.62	2.86	3.17	3.33	3.48	3.55	3.78
	2-methylnaphthalene	2.61	2.88	3.01	3.43	3.25	3.55	3.61	3.98
	C2-naphthalene	3.44	3.64	4.18	4.48	4.47	4.41	4.82	4.80
	C3-naphthalene	3.46	3.53	4.34	4.76	4.28	4.05	4.74	4.67
	C4-naphthalene	3.65	3.89	4.49	5.02	4.24	4.04	4.80	4.69
	C1-fluorene	3.23	3.29	3.86	4.40	3.81	3.64	4.19	4.16
	C2-fluorene	3.87	3.84	4.63	5.12	4.35	4.08	4.83	4.70
	C3-fluorene	–	–	4.51	4.87	4.09	3.86	4.59	4.58
	1-methylphenanthrene	3.48	3.57	4.33	4.81	3.90	3.86	4.44	4.47
	2-methylphenanthrene	3.54	3.63	4.37	4.83	3.92	3.83	4.42	4.44
	3-methylphenanthrene	3.45	3.55	4.27	4.71	3.85	3.80	4.35	4.35
	4/9-methylphenanthrene	3.60	3.65	4.35	4.81	3.99	3.89	4.50	4.49
	C2-phenanthrene	4.00	3.98	4.75	5.03	4.31	4.34	4.84	4.81
	C3-phenanthrene	3.85	3.82	4.43	4.62	4.53	4.52	4.93	5.01
	C4-phenanthrene	-	3.84	4.38	4.27	4.65	4.32	4.80	4.88
	1-methyldibenzothiophene	3.58	3.61	4.40	4.82	3.85	3.85	4.45	4.53
	2/3-methyldibenzothiophene	3.54	3.61	4.40	4.85	3.83	3.82	4.40	4.49
	4-methyldibenzothiophene	3.58	3.66	4.42	4.88	3.83	3.82	4.41	4.50
	C2-dibenzothiophene	4.08	4.10	4.96	5.30	4.25	4.26	4.87	4.91
	C3-dibenzothiophene	3.80	3.79	4.54	4.77	4.19	4.22	4.84	4.77
	C1-chrysene	–	–	–	–	–	–	–	–
	C2-chrysene	–	–	–	–	–	–	–	–
	C3-chrysene	–	–	–	–	–	–	–	–

[a]Values of concentration in fish embryos were normalized by lipid content.

differences in sensitivity. The concentration ratio (C_{embryo}/C_w) observed for flounder and sea bass both suggests key differences in the AhR and CYP1A pathway between these species and provides new information on the identities of specific PAH homologues producing developmental cardiotoxicity.

Flounder embryos accumulated more PAHs than did sea bass, although this result is not uniform across all homologues, and specific compounds accumulated at much higher concentrations (Table 4). With their smaller size and greater surface-to-volume ratio, it would be expected that flounder embryos would accumulate PAHs more rapidly than those of sea bass. In addition, the absorption capacity of embryonic flounder might be saturated in a relatively shorter exposure time because of lower lipid content. However, the weaker CYP1A activity also suggests the sensitivity of flounder may be additionally influenced by either a reduced AhR responsiveness, lower CYP1A metabolic activity, or both. These combined effects of more rapid uptake, faster saturation, and weaker metabolic activity appears to results in greater sensitivity of flounder embryos to developmental toxicity. The lower levels of CYP1A mRNA in response to a higher embryo burden suggests that flounder AhR isoforms are not activated as effectively by the ligands present in IHCO effluent. At the same time, the selectively higher C_{embryo}/C_w ratio for some compounds, in particular the methylated dibenzothiophenes, suggests that flounder CYP1A isoform(s) have reduced enzymatic activity for these specific PAHs. Importantly, the C_{embryo}/C_w ratios for some compounds (e.g., the fluorenes) were essentially identical for flounder and sea bass. This finding also suggests that the dibenzothiophene family members in IHCO could be linked to

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the greater toxicity observed in flounder. In general, these compounds are among the least-characterized petrogenic PAHs.

Although both of these marine species, like zebrafish, demonstrated a similar cardiotoxic response to IHCO as that described for other species and crude oils, our results also support evidence for noncardiac toxic effects distinct to IHCO. The effect of IHCO on these marine species is particularly consistent with our previous results in zebrafish in relation to finfold defects. Embryos exposed to IHCO show more severe fin defects than those exposed to other crude oils (e.g., ANSCO or MC252 crude oil). Although activation of the AhR by potent ligands such as TCDD block regenerative fin growth, potentially through AhR-dependent cross-talk between signaling pathways[30,31] the etiology of this defect in IHCO-exposed embryos is unknown. However, the frequency of fin defects did not correlate with the expression pattern of AhR in flounder and sea bass, arguing against an AhR-dependent pathway.

Therefore, these studies demonstrate that IHCO produces severe cardiotoxicity in two marine species potentially impacted by the *Hebei Spirit* oil spill. The sensitivity of spotted sea bass and olive flounder is in the range of sensitivities observed across a diversity of teleosts, with effects occurring at dissolved total PAH concentrations at 10 μg/L and below. Hence, the combined effects of higher accumulation and weaker CYP1A induction appear to result in different sensitivity for developmental toxic effects between embryonic flounder and sea bass. Importantly, these studies also shed new light on mechanisms that might underlie differences in species sensitivity, highlighting in particular a potential role for differences in protective metabolism by CYP enzymes. Past studies on substrate specificity for CYP1A have focused largely on carcinogenic high-molecular-weight PAHs. Our results here indicate that a greater understanding of oil spill risks may be obtained from toxicokinetic studies of petrogenic PAHs in the early life-history stages of a wider range of fish species.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b03729.

PAH concentrations (ng/L) in oiled gravel effluent of Iranian heavy crude oil) (Table S1), PAH concentrations (ng/g lipid weight) in embryonic fish (Table S2), concentration ratios for selected individual PAHs after 24 h exposure (Table S3), and malformation images in embryonic fish (Figure S1) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +82 (55)639-8680/8689; e-mail: jungjh@kiost.ac.

Notes

The authors declare no competing financial interest.

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