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Species-Specific Metabolism of Naphthalene and Phenanthrene in 3 Species of Marine Teleosts Exposed to Deepwater Horizon Crude Oil

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SPECIES-SPECIFIC METABOLISM OF NAPHTHALENE AND PHENANTHRENE IN 3 SPECIES OF MARINE TELEOSTS EXPOSED TO *DEEPWATER HORIZON* CRUDE OILERIN L. PULSTER,^{a,*} KEVAN MAIN,^b DANA WETZEL,^b and STEVE MURAWSKI^a^aCollege of Marine Science, University of South Florida, St. Petersburg, Florida, USA^bMote Marine Laboratory, Sarasota, Florida, USA

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Abstract: The 2 most abundant polycyclic aromatic hydrocarbons (PAHs) measured in *Deepwater Horizon* crude oil, naphthalene and phenanthrene, and their associated homologs have both been shown to be acutely toxic in fish. Although fish have a relatively high metabolic capacity for PAHs, hydroxylated PAH (OH-PAH) derivatives formed during the initial metabolic response can negatively impact the health of fish. Species-specific metabolism of naphthalene and phenanthrene was evaluated in 3 marine teleosts, red drum (*Sciaenops ocellatus*), Florida pompano (*Trachinotus carolinus*), and southern flounder (*Paralichthys lethostigma*). Fish were exposed to *Deepwater Horizon* crude oil by intraperitoneal injections at time 0 and 48 h, with bile sampling events at 24 and 72 h post injection. The data suggested metabolic induction in Florida pompano and red drum, whereas southern flounder may have demonstrated metabolic fatigue. By 24 h post injection, overall profiles of red drum and southern flounder were dominated by hydroxylated phenanthrene metabolites; conversely, the Florida pompano profiles were dominated by monohydroxylated naphthalenes. In addition, Florida pompano had faster overall relative biotransformation rates, suggesting their potential decreased susceptibility to adverse effects. Red drum and southern flounder had much lower relative biotransformation rates, indicating their probable susceptibility to adverse outcomes after naphthalene and phenanthrene exposures. To our knowledge, the present study is the first to investigate monohydroxylated PAHs in fish exposed to *Deepwater Horizon* oil. *Environ Toxicol Chem* 2017;36:3168–3176. © 2017 The Authors. Environmental Toxicology and Chemistry published by Wiley Periodicals, Inc. on behalf of SETAC.

Keywords: Monohydroxylated polycyclic aromatic hydrocarbons Metabolites Phenanthrene Naphthalene *Deepwater Horizon*

INTRODUCTION

The largest oil spill in US history occurred during 2010 as a result of the massive deep-water blowout at the Macondo wellhead following the sinking of the British Petroleum *Deepwater Horizon* drilling platform in the Gulf of Mexico. The *Deepwater Horizon* incident released an estimated 4.9 million barrels of light sweet crude oil, which contained a higher proportion of lower molecular weight hydrocarbons than other heavier crudes (e.g., the *Exxon Valdez*) and consisted of approximately 3.9% polycyclic aromatic hydrocarbons (PAHs) [1,2]. Two low-molecular-weight PAHs, naphthalene and phenanthrene, and their corresponding homologs represent approximately 74% and 22%, respectively, of the PAHs measured in the oil collected directly from the Macondo well during response operations (MC252 SRM 2779; National Institute of Standards and Technology).

Although PAHs comprise a small compositional percentage of crude oil, they are considered the most toxic component mainly because of their metabolites [3]. The toxic potential of PAHs, in general, and specifically from the *Deepwater Horizon*-impacted waters, have been well documented in fish displaying numerous adverse acute and chronic effects, including skin lesions, cardiotoxicity, liver abnormalities, respiration changes, reduced fecundity, histopathological

changes, and mortality [4–11]. Naphthalene and phenanthrene, the 2 most abundant PAH parent compounds measured in the *Deepwater Horizon* crude oil, have both been shown to be acutely toxic in fish at concentrations ranging from 0.51 to 7.9 mg/L and 0.23 to 1.15 mg/L, respectively [12–16]. In fact, phenanthrene has been found to be one of the most toxic aromatic compounds in petroleum [17].

The primary location of PAH metabolism in fish occurs in the liver, resulting in metabolites that are ultimately stored within the gallbladder for excretion [18]. Fish have a relatively high capacity to rapidly metabolize PAHs because of their high levels of cytochrome P-450 enzymes, which biotransform the parental PAHs into their hydroxylated derivatives (phase I metabolism), followed by the more water-soluble conjugates (phase II metabolism), which are rapidly excreted mainly through the digestive tract and urine [19–22]. However, metabolites can be hydrolyzed, reabsorbed through the gut, and returned through the enterohepatic circulation to the liver, thereby increasing their potential for toxicity [23]. The hydroxylated PAH (OH-PAH) derivatives formed during the initial metabolic response have reactive functional groups (e.g., –OH, –COOH, –NH₂), thus creating more reactive electrophilic compounds that can exhibit carcinogenicity and increase DNA adduct formation, reactive oxygen species, and oxidative stress and can disrupt the synthesis of reproductive hormones in fish [24–27].

Chemical bioavailability, uptake, and biotransformation and excretion rates tend to be a complex function of physicochemical properties (e.g., molecular weight and octanol–water partition coefficient [*K_{ow}*]), species-specific life histories (e.g., trophic level, habitat, and diet), and metabolic capacity. Differences in utilization of biotransformation mechanisms and pathways have been observed between species as well among individual fish

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within a species, yet it is still unclear whether these mechanistic differences are inherently derived or occur as a result of factors related to life history [28]. Willett et al. [29] noted similar conjugation patterns and elimination levels of benzo[*a*]pyrene between 2 related species of ictalurid catfish; however, 1 species had higher levels of biotransformation enzymes (i.e., epoxide hydrolase [EH] and glutathione-S-transferase [GST]), resulting in the preferential formation of a DNA-reactive precursor in that species. After the *Deepwater Horizon* event, significant differences in biliary naphthalene metabolite concentrations were observed among 3 species of demersal fish with different life histories [30]. Understanding the differences in species-specific metabolism and factors related to different life histories is critical in identifying vulnerable and susceptible species that may be prone to various diseases as a result of anthropogenic insults.

The aim of the present study was to evaluate the species-specific metabolism of the 2 most abundant PAHs, naphthalene and phenanthrene, in the *Deepwater Horizon* crude oil in 3 marine teleosts representing different life histories and important recreational fisheries. Red drum (*Scianops ocellatus*), Florida pompano (*Trachinotus carolinus*), and southern flounder (*Paralichthys lethostigma*) were chosen, to represent 3 different life histories—estuarine, pelagic, and benthic, respectively. Fish were exposed to *Deepwater Horizon* crude oil by intraperitoneal injection, and select monohydroxylated metabolites of naphthalene and phenanthrene were identified and quantified in bile using gas chromatography triple quadrupole mass spectrometry.

MATERIALS AND METHODS

Materials

All sample glassware was detergent washed, solvent rinsed, and combusted at 500 °C in a muffle furnace (IsoTemp; Thermo Scientific) overnight prior to use, following general standard operating procedures for trace organics [31]. Optima-grade solvents were purchased from Fisher Scientific. Hydroxylated naphthalene (1-naphthol and 2-naphthol), 9-phenanthrol, 1-triphenylamine (TPA), β -glucuronidase/arylsulfatase (from *Helix pomatia*), and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Sigma-Aldrich; 1-Phenanthrol, 2-phenanthrol, 3-phenanthrol, and 4-phenanthrol were purchased from Cambridge Isotope Laboratories; and tricaine-methanesulfonate (MS-222) was purchased from Western Chemical.

Experimental fish

Red drum (*S. ocellatus*, $n = 10$), Florida pompano (*T. carolinus*, $n = 10$), and southern flounder (*P. lethostigma*, $n = 10$) were chosen to represent 3 different life histories—estuarine, pelagic, and benthic, respectively. The red drum and pompano were produced from captive spawned broodstock at Mote Aquaculture Research Park (Sarasota, FL, USA). Southern flounder were produced from captive spawned broodstock at Texas Parks and Wildlife Department (Corpus Christi, TX, USA) and reared to 30 d of age prior to shipping to Mote Aquaculture Research Park. All fish were reared at Mote Aquaculture Research Park until they were subadults; specific ages were 14, 11, and 18 mo old for red drum, pompano, and southern flounder, respectively. Fish were fasted and kept in separate 90-L containers and monitored for temperature, pH (day 2 only), salinity, and dissolved oxygen (see the Supplemental Data) for the duration of the 72-h exposures. All exposures were conducted at Mote Aquaculture Research Park. Sample sizes were constrained to the limitations of the complex nature of the present study because of

the relatively large size of the fish used (compared with typical model species, i.e., Cypriniformes) and the large aquaculture support facility necessary to maintain the fish.

Experimental treatments and sampling

Treatment concentrations and protocols were adapted from methods of Celander et al. [32] and Carlson et al. [33]. Treatments were produced in duplicate for each of the 3 species and consisted of blank controls (no treatment), corn oil controls, time-zero injection, and a reinjection at 48 h (Table 1). Subadult fish of each of the 3 species were anesthetized with MS-222 at 300 mg/L and then exposed by intraperitoneal injection with either corn oil (control) or 2 mg/kg unweathered *Deepwater Horizon* crude oil (MC252 SOB-20100628-050 and SOB-20100628-047) in corn oil (Sigma-Aldrich) at a 2:1 ratio (crude:corn oil). Following injection, fish were revived in aerated tanks before transfer to individual containers for the duration of the experiment. Control fish with no treatment were euthanized at time zero, whereas the intraperitoneal exposed fish were euthanized at 24 h post intraperitoneal injection and at 72 h (post reinjection at 48 h). Fish were euthanized using MS-222 at 300 ppm for 15 min. During dissections, the gallbladder was excised and fully drained into a clean amber vial and stored at -20 °C until further analysis.

Enzymatic hydrolysis and derivatization

Enzymatic treatment was based on modified methods by Willett et al. [29] and Krahn et al. [34]. Briefly, prior to analysis, bile (100 μ L) was deconjugated enzymatically by a 24-h incubation at 40 °C with β -glucuronidase and aryl-sulfatase (200 μ L) in 1 mL sodium acetate buffer (pH 5). Following incubation, 1 mL of ethyl acetate:acetone (2:1 ratio) was added, and then the bile was mixed, vortexed, and centrifuged (4000 rpm for 5 min). The organic phase was transferred to a clean vial, and the liquid extraction was repeated 2 additional times with ethyl acetate. Organic fractions were combined and blown down to dryness under ultra-high-purity nitrogen. Residues were redissolved in 100 μ L of BSTFA and incubated at 70 °C for 1 h. Residues were then redissolved with an internal standard (TPA) and hexane for a final extract volume of 100 μ L.

GC-MS/MS analysis

Extracts were injected (2 μ L) onto an Agilent 7890B gas chromatograph (GC) coupled to a 7010 tandem mass spectrometer (MS/MS) operating in electron impact and multiple reactions mode (see Table 2 for parameters). The multimode inlet and source temperatures were set at 250 and 230 °C, respectively. Chromatographic separation was achieved using a Restek-5Sil with an Integra-guard column (30 m \times 250 μ m \times 0.25 μ m; Restek) using ultra-high-purity helium as the carrier gas. The initial GC oven temperature of 120 °C was held for 2 min and then increased to 200 °C at 10 °C/min, followed by a 5 °C/min increase to 250 °C, and a 15 °C/min increase to a final temperature of 300 °C with a 3-min hold, resulting in a total run time of 26.33 min. The MS/MS collision cell used ultra-high-purity nitrogen as the collision-induced dissociation gas, with a flow rate of 1.5 mL/min and ultra-high-purity helium as the quench gas with a flow rate of 2.25 mL/min.

Selected monohydroxylated metabolites were identified by standards, retention time, and quantifier and qualifier ion peak ratios. Sample spectra were scanned for fragmentation patterns of the BSTFA derivatizations and their subsequent trimethylsilyl (TMS) groups and monitored for the presence of peaks at m/z 73, corresponding to the excess derivatization reagent indicating a complete reaction. Metabolite quantitation in the

Table 1. Biometrics, treatment type, and injection volumes used during the present 72-h exposure study

Species	Fish ID	Weight (kg)	Length (mm)	Sex	Exposure type	Injection Time (T) and Treatment			
						T = 0 h Crude oil (mL)	T = 0 h Corn oil (mL)	T = 48 h Crude oil (mL)	T = 48 h Corn oil (mL)
RD	RF1	0.755	390	Male	Control	—	—	—	—
RD	RF2 ^a	0.470	340	Male	Control	—	—	—	—
RD	RF3	1.213	480	Female	Corn oil	—	1.20	—	—
RD	RF4	0.649	391	Female	Corn oil	—	0.65	—	—
RD	RF5	1.205	443	Unknown	Corn oil	—	1.20	—	1.20
RD	RF6	0.866	413	Male	Corn oil	—	0.87	—	0.87
RD	RF7	0.599	353	Male	Crude and corn oil	1.20	0.60	—	—
RD	RF8	0.919	422	Female	Crude and corn oil	1.80	0.92	—	—
RD	RF9	0.866	423	Male	Crude and corn oil	1.80	0.89	1.80	0.89
RD	RF10	0.907	412	Male	Crude and corn oil	1.80	0.91	1.80	0.91
FP	FP1	0.373	316	Female	Control	—	—	—	—
FP	FP2	0.467	361	Male	Control	—	—	—	—
FP	FP3	0.391	283	Male	Corn oil	—	0.39	—	—
FP	FP4	0.382	313	Male	Corn oil	—	0.38	—	—
FP	FP5	0.399	332	Unknown	Corn oil	—	0.40	—	0.40
FP	FP6	0.379	325	Female	Corn oil	—	0.38	—	0.38
FP	FP7	0.461	339	Male	Crude and corn oil	0.92	0.46	—	—
FP	FP8	0.576	341	Female	Crude and corn oil	1.00	0.52	—	—
FP	FP9	0.329	307	Male	Crude and corn oil	0.66	0.33	0.66	0.33
FP	FP10	0.354	298	Male	Crude and corn oil	0.70	0.35	0.70	0.35
SF	SF1	0.188	240	Unknown	Control	—	—	—	—
SF	SF2 ^a	0.212	250	Unknown	Control	—	—	—	—
SF	SF3 ^a	0.319	298	Unknown	Corn oil	—	0.32	—	—
SF	SF4 ^a	0.223	268	Unknown	Corn oil	—	0.22	—	—
SF	SF5 ^b	—	—	Unknown	Corn oil	—	0.28	—	0.28
SF	SF6 ^b	—	—	Unknown	Corn oil	—	0.15	—	0.15
SF	SF7	0.372	297	Unknown	Crude and corn oil	0.74	0.37	—	—
SF	SF8 ^c	—	—	Unknown	Crude and corn oil	0.40	0.20	—	—
SF	SF9 ^b	—	—	Unknown	Crude and corn oil	0.46	0.23	0.46	0.23
SF	SF10 ^b	—	—	Unknown	Crude and corn oil	0.48	0.24	0.48	0.24

^aNo bile.^bDead at 72 h.^cDead at 24 h.

RD = red drum; FP = Florida pompano; SF = southern flounder.

enzymatically treated samples was conducted by direct ratio comparisons of the target analytes to the internal standard, TPA. A data quality control and assurance program was in place to ensure samples passed acceptable criteria. Matrix and blanks were spiked with 9 monohydroxylated PAHs with mean recoveries passing valid quality control criteria (mean 80–120%). There were no detectable levels observed in the control fish or the corn oil controls. Validation was achieved using linear calibration curves for silanized standards for all compounds, with an acceptance calibration criteria of $\pm 20\%$ relative standard deviation for all average relative response factors. The method detection limit of 750 pg/mL is defined as the lowest standard with all detectable levels of target analytes.

Data analysis

As a result of mortalities or lack of bile at time of collections, final sample sizes were adjusted accordingly (Florida pompano $n = 10$; red drum $n = 9$; southern flounder $n = 2$) for metabolite analysis (Table 1). The evacuation of bile is stimulated by digestive and nutritional signals. Because of the episodic nature, bile is not always found. Table 3 shows individual fish data, expressed as ng/mL. The sums of all 7 OH-PAHs (total biliary concentrations) are given for a species at a specific time point (i.e., 24 or 72 h) and are represented as the \sum_7 OH-PAHs. The sum of the 2 monohydroxylated naphthalene compounds is expressed as \sum_2 OH-Nap. The sum of the 5 monohydroxylated phenanthrene compounds is expressed as \sum_5 OH-Phn. The

Table 2. Analytical parameters for analysis of OH-Nap and OH-Phn in bile using GC-MS/MS

Analyte	RT (min)	Quantification transition	CE (V)	Confirmation transition	CE (V)
1-OH-Nap	8.6	201→185	30	216→185	30
2-OH-Nap	8.9	216→185	30	201→185	30
TPA (IS)	14.7	245→243	20	245→165	20
4-OH Phn	15.2	266→235	25	251→235	30
9-OH Phn	15.8	251→235	30	266→235	25
3-OH Phn	16.2	251→176	30	266→251	25
1-OH Phn	16.3	266→235	25	251→235	30
2-OH Phn	16.8	251→176	30	266→251	25

OH-Nap = hydroxylated naphthalene; OH-Phn = hydroxylated phenanthrene; GC-MS/MS = gas chromatography–tandem mass spectrometry; RT = retention time; CE = collision energy; TPA = 1-triphenylamine; IS = internal standard.

Table 3. Biliary concentrations for select monohydroxylated metabolites and metabolite contributions in Florida pompano, red drum, and southern flounder after intraperitoneal injections of 2 mg/kg of Deepwater Horizon crude oil

Fish ID	Treatment Sex	Florida pompano						Red drum						Southern flounder							
		0 h		24 h		72 h		0 h		24 h		72 h		0 h	24 h						
		Control F/M	CoC M	Oil exposure M	FP7 F	FP8 F	CoC U/F	Oil exposure M	FP9 M	FP10 M	Control M	RF3 (F) F	RF4 (F) F	Oil exposure M	RF5 (U) U/M	RF6 (M) M	RF7 M	RF8 M	RF9 M	RF10 M	Control U
1-OH-Nap	<MDL	<MDL	5.1	6.2	280	<MDL	280	1412	<MDL	<MDL	<MDL	3.9	<MDL	<MDL	<MDL	81	170	<MDL	<MDL	<MDL	2.5
2-OH-Nap	<MDL	<MDL	15	12	303	<MDL	303	500	<MDL	<MDL	<MDL	4.6	<MDL	<MDL	<MDL	50	120	<MDL	<MDL	<MDL	5.0
1-OH-Phn	<MDL	<MDL	1.1	7.0	680	<MDL	680	970	<MDL	<MDL	<MDL	39	<MDL	<MDL	100	140	50	<MDL	<MDL	<MDL	33
2-OH-Phn	<MDL	<MDL	<MDL	1.5	146	<MDL	146	340	<MDL	<MDL	<MDL	9.1	<MDL	<MDL	36	46	38	<MDL	<MDL	<MDL	29
3-OH-Phn	<MDL	<MDL	<MDL	0.8	91	<MDL	91	230	<MDL	<MDL	<MDL	10	<MDL	<MDL	34	46	38	<MDL	<MDL	<MDL	17
4-OH-Phn	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	92	150	<MDL	<MDL	<MDL	4.2	<MDL	<MDL	18	38	47	<MDL	<MDL	<MDL	8.9
9-OH-Phn	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	4.7	<MDL	<MDL	<MDL	9.7
∑ ₂ OH-Nap	<MDL	<MDL	20	18	586	<MDL	586	1900	<MDL	<MDL	<MDL	8.5	<MDL	<MDL	131	290	290	<MDL	<MDL	<MDL	7.5
∑ ₅ OH-Phn	<MDL	<MDL	1.1	9.4	1006	<MDL	1006	1700	<MDL	<MDL	<MDL	62	<MDL	<MDL	190	280	280	<MDL	<MDL	<MDL	98
∑ ₇ OH-PAH	<MDL	<MDL	21	27	1600	<MDL	1600	3600	<MDL	<MDL	<MDL	71	<MDL	<MDL	320	570	570	<MDL	<MDL	<MDL	106
Metabolite contributions (%)																					
1-OH-Nap	<MDL	<MDL	24	23	18	<MDL	18	39	<MDL	<MDL	<MDL	5.5	<MDL	<MDL	26	30	30	<MDL	<MDL	<MDL	2.3
2-OH-Nap	<MDL	<MDL	71	43	19	<MDL	19	14	<MDL	<MDL	<MDL	6.5	<MDL	<MDL	16	21	21	<MDL	<MDL	<MDL	4.7
1-OH-Phn	<MDL	<MDL	5	26	43	<MDL	43	27	<MDL	<MDL	<MDL	55	<MDL	<MDL	31	24	24	<MDL	<MDL	<MDL	31
2-OH-Phn	<MDL	<MDL	<MDL	6	9.2	<MDL	9.2	9.4	<MDL	<MDL	<MDL	13	<MDL	<MDL	11	8.8	8.8	<MDL	<MDL	<MDL	28
3-OH-Phn	<MDL	<MDL	<MDL	3	5.7	<MDL	5.7	6.3	<MDL	<MDL	<MDL	14.6	<MDL	<MDL	11	8.1	8.1	<MDL	<MDL	<MDL	16
4-OH-Phn	<MDL	<MDL	<MDL	<MDL	5.8	<MDL	5.8	4.2	<MDL	<MDL	<MDL	5.9	<MDL	<MDL	5.6	6.7	6.7	<MDL	<MDL	<MDL	8.5
9-OH-Phn	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	0.8	0.8	<MDL	<MDL	<MDL	9.2
∑ ₂ OH-Nap	<MDL	<MDL	95	65	37	<MDL	37	53	<MDL	<MDL	<MDL	12	<MDL	<MDL	41	51	51	<MDL	<MDL	<MDL	7
∑ ₅ OH-Phn	<MDL	<MDL	5	35	63	<MDL	63	47	<MDL	<MDL	<MDL	88	<MDL	<MDL	59	49	49	<MDL	<MDL	<MDL	93

CoC = corn oil controls; F = female; M = male; U = unknown; OH-Nap = hydroxylated naphthalene; OH-Phn = hydroxylated phenanthrene; ∑₂OH-Nap = 1-OH-Nap + 2-OH-Nap; ∑₅OH-Phn = 1-OH-Phn + 2-OH-Phn + 3-OH-Phn + 4-OH-Phn + 5-OH-Phn; ∑₇OH-PAH = 1-OH-PAH + 2-OH-PAH + 3-OH-PAH + 4-OH-PAH + 5-OH-PAH + 6-OH-PAH + 7-OH-PAH; MDL = method detection limit.

mean percent contributions for each species and time period (24 and 72 h) were used for species comparisons. Mean concentrations (ng/mL) were used for comparison purposes; however, the small sample sizes necessitate interpretive caution. Because of mortalities and lack of bile, results are shown for only 2 southern flounder (1 control, 1 exposed); therefore, these were not included in species comparisons. All data analysis was performed using JMP 13 (SAS Institute) with a significance level of $p < 0.05$. If data failed assumptions of normality, nonparametric hypotheses tests were run to evaluate differences between treatments, and time variations within a species, to evaluate potential explanatory variables between species and all pairwise post hoc multiple comparisons using Kruskal–Wallis one-way analysis of variance on ranks followed by Tukey's test or Dunn's test or Spearman's test.

RESULTS

Total biliary OH-PAH ($\sum_7\text{OH-PAH}$) concentrations

Biliary levels and patterns of the select monohydroxylated naphthalenes and phenanthrenes were assessed in Florida pompano, red drum, and southern flounder (Table 3). The total concentrations of detectable levels of select monohydroxylated PAHs ($\sum_7\text{OH-PAH}$) measured in the present study ranged from 21 to 3600 ng/mL. At 24 h post injection, the southern flounder (106 ng/mL) and red drum (71–120 ng/mL) had similar $\sum_7\text{OH-PAH}$ s, with levels up to 5 times higher than Florida pompano (21–27 ng/mL; Figure 1). Although the levels had not yet reached equilibrium by 72 h, concentrations increased up to 1 and 2 orders of magnitude in the red drum and pompano, respectively. At 72 h, mean $\sum_7\text{OH-PAH}$ concentrations measured in Florida pompano (2600 ng/mL) were a factor of 6 times higher than mean levels in red drum (445 ng/mL; Figure 1). Only 24-h measurements were possible in the southern flounder because of 100% mortality by 72 h.

Monohydroxylated $\sum_2\text{OH-NAP}$ and $\sum_5\text{OH-PHN}$ metabolite concentrations and contributions

The concentrations and relative contributions of the specific hydroxylated naphthalene and phenanthrene metabolites

detected in the 3 species in the present study are shown in Table 3. The monohydroxylated naphthalene and phenanthrene metabolite concentrations in the Florida pompano increased over time. For instance, by 72 h the mean $\sum_2\text{OH-Nap}$ (1200 ng/mL) and $\sum_5\text{OH-Phn}$ (1400 ng/mL) concentrations were 63 and >200 times higher than those measured at 24 h (19 ng/mL $\sum_2\text{OH-Nap}$; 5.3 $\sum_5\text{OH-Phn}$). The monohydroxylated naphthalene metabolites predominated at 24 h ($80 \pm 20\%$); however, by 72 h, the hydroxylated phenanthrene metabolites ($55 \pm 12\%$) marginally dominated the profiles in the Florida pompano bile (Figure 2).

Similar increases in concentrations over time were also observed in the red drum. Mean biliary monohydroxylated naphthalene concentrations at 24 h (16 ng/mL) increased by a factor of 13 by 72 h (210 ng/mL). Mean monohydroxylated phenanthrene metabolites in red drum increased by a factor of 3 between 24 h (78 ng/mL) and 72 h (240 ng/mL). In contrast to the Florida pompano profiles, the red drum 24-h bile samples were dominated by the monohydroxylated phenanthrene metabolites ($84 \pm 5\%$). By 72 h, the metabolite contributions were similar to the Florida pompano profiles, with mean monohydroxylated phenanthrene metabolites accounting for $54 \pm 7\%$, whereas the monohydroxylated naphthalene metabolites accounted for $46 \pm 7\%$ (Figure 2).

Similar to the red drum, the targeted monohydroxylated phenanthrenes predominated in the southern flounder bile, and monohydroxylated phenanthrene concentrations (98 ng/mL) accounted for 93% of the biliary metabolites measured at 24 h and were almost 5 times higher than the monohydroxylated naphthalene concentrations (7.5 ng/mL; Table 3 and Figure 2). The concentrations measured in southern flounder ranged from 2.5 to 33 ng/mL for 1-naphthol and 1-phenanthrol, respectively; 1-phenanthrol was followed by 2-phenanthrol, 3-phenanthrol, 9-phenanthrol, 4-phenanthrol, 2-naphthol, and 1-naphthol. Interestingly, southern flounder was the only species with detectable levels of 9-phenanthrol at 24 h.

Biotransformation rates

Because of the variation between individuals and species combined with small sample sizes, the following biotransformation rates presented require interpretive caution; however, it

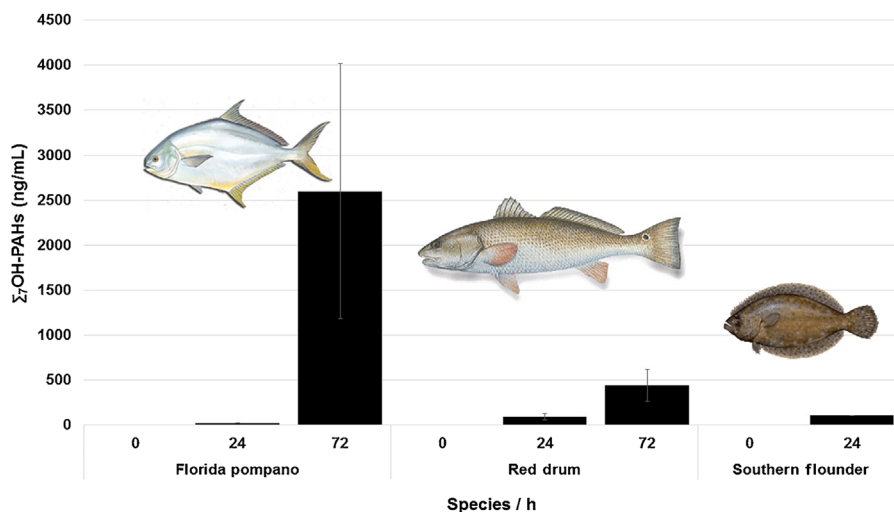


Figure 1. Mean concentrations of the total select monohydroxylated metabolites ($\sum_7\text{OH-PAHs}$ ng/mL) in Florida pompano, red drum, and southern flounder at 24 and 72 h. Although concentrations had not yet reached equilibrium by 72 h, concentrations increased by 1 and 2 orders of magnitude in the red drum and Florida pompano, respectively. All controls and corn oil controls for each species were below the method detection limit, and therefore are represented by one time zero control. Error bars represent standard deviations between the 2 treatment fish.

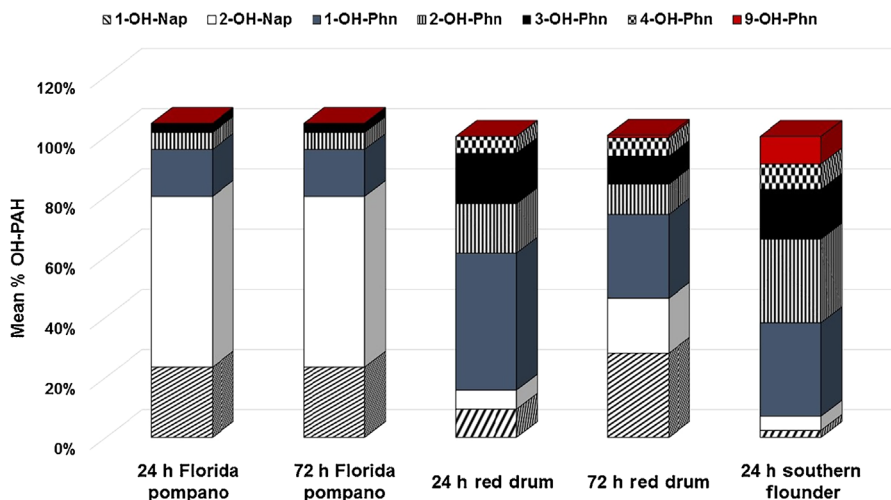


Figure 2. Mean metabolite contributions (%) of the individual monohydroxylated naphthalenes (OH-Nap) and phenanthrenes (OH-Phn) measured in the bile of each species over time.

is important to discuss these data in terms of the observed differences in results between species.

Interestingly, the 2 targeted monohydroxylated naphthalene metabolites were the most abundant metabolites ($78 \pm 19\%$) in Florida pompano after 24 h, demonstrating the preferential metabolism and higher biotransformation rate compared with the 5 selected monohydroxylated phenanthrene metabolites. Evaluation of the relative biotransformation rates ($\text{ng/mL/h} \times \% \text{ metabolite}$) for each metabolite revealed that indeed the mean 24-h relative biotransformation rates for the $\sum_2\text{OH-Nap}$ metabolites in Florida pompano (0.37 ng/mL/h) were up to an order of magnitude higher than that for the mean of $\sum_5\text{OH-Phn}$ metabolites (0.04 ng/mL/h) as well as compared with those rates in red drum and southern flounder (Figure 3). Furthermore, by 72 h the mean relative biotransformation rates in Florida pompano for the 2 monohydroxylated naphthalene metabolites (5.08 ng/mL/h) and phenanthrene metabolites (4.35 ng/mL/h) were 14 times and almost 100 times higher, respectively, than those measured at 24 h. In addition, the 72-h pompano naphthalene and phenanthrene relative biotransformation rates were 14 and 4 times faster, respectively, than those rates observed in red drum at 72 h.

Conversely, the relative biotransformation rates for phenanthrene were higher than those for naphthalene in both the red drum and southern flounder. In red drum, the relative biotransformation rate for phenanthrene at 24 h was 8 times faster than that for naphthalene and 2 orders of magnitude higher than the phenanthrene biotransformation rates in Florida pompano. The naphthalene relative biotransformation rates in red drum demonstrated a 20-fold increase over time, yet the median phenanthrene relative biotransformation rates decreased by half between 24 h (0.118 ng/mL/h) and 72 h (0.052 ng/mL/h). Similar to the red drum, the overall median phenanthrene relative biotransformation rate in southern flounder at 24 h was almost 80 times faster than that for naphthalene and >900 times higher than those observed in Florida pompano.

DISCUSSION

Although all 3 species of fish received 2 injections of *Deepwater Horizon* crude oil (2 mg/kg) at time 0 and repeated at 48 h, 100% mortality within 72 h was noted solely in southern flounder. This finding in flounder may be the result of injection

injury, stress, metabolic fatigue, or toxicity. Southern flounder have a very small and tightly compact body cavity, which can make intraperitoneal injections challenging. Although there was no obvious evidence of internal injury at the time of dissections, this factor cannot be ruled out as a source of mortality in this species, considering that 2 of the 4 corn oil controls also died within 72 h. Toxicity and metabolic fatigue also could have resulted in the observed mortalities. A recent study found 100% mortality of southern flounder exposed to 395 mg/kg tPAH50 (sum of 50 individual PAH concentration measurements) *Deepwater Horizon*-contaminated sediments after 18 d [5]. Furthermore, those authors [5] calculated the median lethal concentration for their 32-d sediment exposure to be 78 mg/kg

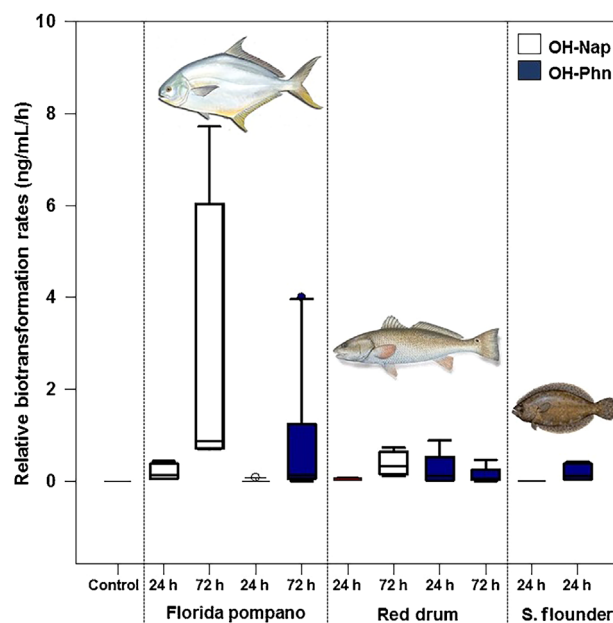


Figure 3. Relative biotransformation rates (ng/mL/h) for select monohydroxylated naphthalene ($\sum_2\text{OH-Nap}$) and phenanthrene ($\sum_5\text{OH-Phn}$) in bile of Florida pompano, red drum, and southern flounder at 24 h post injection and 72 h post repeat injections. All controls and corn oil controls were below the method detection limit and are therefore represented in this graph as one control.

tPAH50. In contrast, the present study exposed fish to a complex mix, hence toxicity and metabolic fatigue also cannot be ruled out as a source of mortality. In addition, 9-hydroxyphenanthrene (9.7 ng/mL) was only detected in the southern flounder after 24 h. Based on recent evidence indicating that this compound inhibits the biosynthesis of reproductive hormones, southern flounder may suffer from reduced fecundity as a result of exposures to 9-hydroxyphenanthrene [24].

The present study establishes species-specific differences in the naphthalene and phenanthrene metabolism in 3 species of marine teleosts exposed to *Deepwater Horizon* crude oil via intraperitoneal injections. The individual monohydroxylated metabolite profiles measured in the present study varied across individual, species, and time. However, among the monohydroxylated phenanthrene profiles, 1-hydroxyphenanthrene accounted for the highest concentrations in all 3 species. Similarly, 1-hydroxyphenanthrene has been reported to be the highest hydroxylated phenanthrene in other species, including the coalfish, the European flounder (*Platichthys flesus*), rainbow trout (*Salmo gairdneri*), and in mammals [35–37]. These species were exposed to the individual compound phenanthrene via environmental or experimental exposures (i.e., dietary, intragastric, or intraperitoneal). In addition, Kemp's ridley sea turtles collected in the northern Gulf of Mexico during the *Deepwater Horizon* spill had detectable levels of 1-, 2-, and 3-hydroxyphenanthrene, albeit at generally low concentrations (≤ 35 ng/g wet wt) [38]. Thus, these studies, combined with the results from the present study, suggest that 1-hydroxyphenanthrene may be the principle biotransformation product regardless of species, exposure route or whether phenanthrene is introduced as a single compound or within a complex mixture such as crude oil.

Taking into account the repeated injection at 48 h, the rapid increases observed in both pompano and red drum concentrations and biotransformation rates might suggest metabolic induction, considering that petroleum has been known to induce monooxygenase activity in fish [39]. The substantial increase in metabolite concentrations and biotransformation rates in both Florida pompano and red drum after reinjection cannot be solely due to the reinjected dose alone. Instead, this is more symptomatic of a metabolic induction as a result of the repeat doses. Although the levels increased over time, total measured levels in all 3 species only accounted for a small fraction ($< 1\%$) of the theoretical dose of individual naphthalene and phenanthrene in the crude oil intraperitoneal injection. In contrast, other studies reported that 7 to 13% of phenanthrene doses (16 μ g) administered to coalfish intragastrically were present in the gallbladder at 36 to 96 h after dosing [40]. Likewise, 72 h after a single intraperitoneal dose of pyrene (10 mg/kg) in rainbow trout, metabolites accounted for approximately 11% and 41% in the urine and bile, respectively [41]. However, in both of these studies, fish were dosed with a single compound as opposed to a crude oil that contains a complex mixture of thousands of compounds, as in the present study.

In the present study, fish were subject to repeat intraperitoneal injections of *Deepwater Horizon* crude oil (2 mg/kg), corresponding to an average dose of 2500 ± 930 mg of oil per fish, with the individual compound doses being substantially lower for naphthalene ($\sim 2.1 \pm 0.76$ mg/kg) and phenanthrene ($\sim 0.61 \pm 0.23$ mg/kg). Therefore, the low percentage of monohydroxylated metabolites in the bile after 72 h could potentially demonstrate metabolic fatigue or incomplete absorption from being exposed to multiple relatively high doses of crude oil. Alternatively, additional excretion routes and

phase I and phase II biotransformation products cannot be discounted as the dominant pathway. Correspondingly, a major metabolite of phenanthrene (phenanthrene-1,2-dihydrodiol) only accounted for 0.2% of the administered dose after 48 h in coalfish exposed intragastrically to 75 mg/kg, suggesting a potential decreased metabolic efficiency with higher doses [35,37]. The authors also noted that 47% of the dose was located in the intestinal tract as unchanged phenanthrene, supporting the incomplete absorption theory. An unquantified amount of oil was documented in the intraperitoneal cavities of the fish in the present study.

Both life history and exposure routes play significant roles in the bioavailability, uptake, and biotransformation and excretion rates. Moreover, sex differences can also impact differences in body burdens and biotransformation rates, but this is also species-specific. For instance, male fish tend to have higher concentrations of contaminants primarily because of a higher rate of energy expenditure, higher swimming activity, and an increase in mixed oxygenase function activity because of lower circulating estrogens [42–44]. In the present study, there was only 1 female pompano with detectable levels after 24 h, yet these levels were not statistically different from the male pompano at 24 h ($p = 0.12$). In addition, individual variability has been observed and can be the result of a number of physiological parameters such as lipid content, organ weights, and fish weights and lengths, as well as amounts of circulating reproductive and mixed oxygenase function enzymes and hormones.

Although intraperitoneal injections do not reveal any insights into life history factors, they do offer invaluable knowledge in terms of the metabolic capabilities of different species. Nonetheless, with intraperitoneal injections, we can begin to recognize as well as estimate and predict which species may be more susceptible to certain compounds based on their biotransformation capabilities and biotransformation rates. For instance, phenanthrene tends to bind to critical biotransformation enzymes in winter flounder, thereby impacting their detoxification ability and resulting in high incidences of tissue abnormalities [45]. Although the southern flounder in the present study had relatively high biotransformation rates for phenanthrene, they hypothetically suffered from metabolic fatigue and consequently, mortality. Moreover, southern flounder have been shown to have severe adverse effects across multiple endpoints when exposed to environmentally relevant concentrations of *Deepwater Horizon*-contaminated sediment [5,46].

To our knowledge, the present study is the first report of naphthalene and phenanthrene metabolism in these 3 species. In addition, it is also the first report of concentrations of monohydroxylated PAH metabolites in finfish exposed to *Deepwater Horizon* crude oil. The results from the present study demonstrate that the metabolic capacity in Florida pompano and red drum is induced with multiple exposures. Overall, the Florida pompano have faster relative biotransformation rates for naphthalene and phenanthrene, suggesting their potentially decreased susceptibility to adverse effects from these compounds, whereas the red drum and southern flounder had much lower overall relative biotransformation rates, indicating their probable susceptibility to adverse outcomes after exposure to naphthalene and phenanthrene, the 2 most abundant parent PAH compounds found in *Deepwater Horizon* oil. These results suggest the potential for species-specific vulnerability to adverse outcomes exposed to different compounds.

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Data availability—Data are publicly available through the Gulf of Mexico Research Initiative Information & Data Cooperative (GRIIDC) at <https://data.gulfresearchinitiative.org> (doi: 10.7266/N7057CZW).

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