Relating Acute Oil Injury in Topsmelt to PAH Metabolite Concentrations in Bile

Regina Donohoe, Martice Vasquez, and April DaSilva
California Department of Fish and Wildlife, Office of Spill Prevention and Response

Bryn Phillips and Katie Siegler
University of California, Davis

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are toxic components of petroleum and an organism's exposure to these compounds is of concern when an oil spill occurs. Fish rapidly take up PAHs from their environment and metabolize them to more water-soluble compounds that are excreted in the bile (Krahn et al., 1992). Elevated biliary PAH metabolite concentrations have been measured in fish following oil spills (Collier et al., 1997, Jewett et al., 2002, Krahn et al., 1986, Krahn et al., 1992). However, there is a gap in the scientific knowledge regarding the linkage of metabolite concentrations in fish, to the level of exposure, and the subsequent biological effect. The goal of this study was to perform an acute exposure of a representative fish species, the topsmelt (*Atherniops affinis*), to a water accommodated fraction of an offshore Santa Barbara, California crude oil and correlate biliary PAH metabolite concentrations to changes in behavior, such as swimming performance. Oil exposure has been shown to produce narcosis and cardiotoxicity in fish, resulting in reduced swimming speed (Kennedy and Farrell, 2006; Mager et al., 2014; Stieglitz et al., 2016).

An additional component of this study provided an interlaboratory comparison exercise of PAH metabolite quantification in bile using high performance liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS). The National Oceanic and Atmospheric Association (NOAA) Montlake Laboratory at the Northwest Fisheries Science Center previously developed an analytical method for the determination of PAH metabolites in bile (Ylitalo et al., 2017). This approach was duplicated at the California Department of Fish and Wildlife (CDFW) Office of Spill Prevention and Response (OSPR) Petroleum Chemistry Laboratory and sample splits from this study were analyzed by both the NOAA and CDFW laboratories for method comparison, performance, and validation.

This work will provide reference for relating biliary metabolite levels in exposed fish to injury that can be used in a natural resource damage assessment following an oil spill.

Methods

Test organisms

Topsmelt (*Atherinops affinis*) were collected from Elkhorn Slough, California and acclimated in seawater at the University of California, Davis Marine Pollution Studies Laboratory (Granite Canyon, CA). Topsmelt are common in California estuaries and marine waters, and crude oil bioassays have been previously conducted with them at the Granite Canyon Laboratory (Tjeerdema et al., 2010).

Experimental Design

Exposures were conducted with high-energy water accommodated fractions (HEWAFs) of an offshore Santa Barbara, California, crude oil. HEWAF was prepared according to the Deepwater Horizon Laboratory Toxicity Testing Quality Assurance Project Plan (Status Consulting, 2015). Exposures were conducted with both weathered and

unweathered oil. In previous studies at Granite Canyon, weathering was performed by heating the oil to 70ºC until the oil mass was reduced by 20% (Van Scoy et al., 2012).

Topsmelt were exposed for 72 hours under various treatment conditions as described in Table 1. Each treatment consisted of 3 replicate tanks (18 L) with 10 fish in each tank, and a seawater control (3 replicates, 10 fish each). Daily behavior and water quality measurements were recorded. After 72 hours, five fish per replicate were videotaped separately and together. To stimulate a response, stimuli in the form of sound was applied to the exterior of the tank using a bolt attached to a string. Approximately 3 minutes into the recording, the stimuli were applied seven times at 5 second intervals. Once the stimuli were complete, the recording continued to further record fish behavior including fish recovering back to a steady state after being startled. Each fish and replicate groups were recorded for five minutes each. For each replicate, the total number of times each individual fish responded to the stimuli was tallied. That number was summed for each fish in the replicate and compared to the total responses that could result from the stimuli. Following the behavior evaluation, all fish were euthanized, body weight and fork length were measured, and fish were photographed and examined for external lesions.

Table 1. Description of exposure conditions for three different tests using HEWAF prepared from offshore Santa Barbara, California, crude oil.

Parameter	**Conditions**
Test organism	Topsmelt (*Atherinops affinis*)
Source	Elkhorn Slough, California
Life-stage	Adult
Organisms per replicate	10
Exposure chambers	18-liter polycarbonate exposure chambers with lids
Treatments (oil loading)	(1) Static exposure (1 g/L), unweathered oil, with aeration (2) Static exposure (1 g/L) weathered oil and daily renewal exposure (1 g/L) unweathered oil, without aeration (3) Daily renewal exposure (0.5 and 1 g/L) unweathered oil, without aeration
Oil source	offshore Santa Barbara, California crude oil
Temperature	15 ± 1°C
Test duration	72 hours
Observations	Daily behavior and water quality measurements, behavior with stimuli

Fish were dissected to remove the gall bladder, and bile was composited into sample vials by replicate. Composited bile was split for PAH metabolite analysis by the NOAA and CDFW laboratories. Composite water samples were collected from each replicate for each treatment on Day 0 and Day 3 for PAH and TPH analysis. Livers were removed and composited into sample vials by replicate (dead fish livers were frozen, ½ of live fish livers went into formalin and ½ were frozen). The head, including gills, were preserved in formalin for potential histopathology, and carcasses were retained in foil packets. Bile from dead fish were composited by replicate separately.

Chemical Analysis

Analysis of biliary PAH metabolites was performed by both NOAA and CDFW laboratories. The NOAA PAH metabolite analytical method is described in Ylitalo, et al., (2017); this method was the approach used by the CDFW laboratory. Briefly, 50 µL of bile was diluted with an equal volume of water prior to phospholipid removal and subsequent enzymatic hydrolysis of conjugated PAH metabolites into free hydroxylated metabolites. The hydrolyzed extract was cleaned using a hydrophobic-lipophilic balanced sorbent to remove additional sample matrix prior to analysis by LC/MS/MS. A surrogate compound was used to monitor extraction efficiency and to correct results. PAH metabolite lists varied between laboratories, with the NOAA laboratory having a more extensive list. Biliary PAH metabolite profiles for the three treatments in this study are presented using the NOAA laboratory data. CDFW biliary PAH metabolite data is presented with the interlaboratory comparison results. The list of common analytes was used for method comparison. Abbreviations for PAHs and PAH metabolites are listed in Table 2 and Table 3, respectively.

One-liter HEWAF samples were liquid-liquid extracted with dichloromethane and concentrated to a final volume of 1 mL. Half of the 1 mL extract was analyzed for total petroleum hydrocarbons (TPH; diesel range organics (C_{10}-C_{28}) and motor oil range organics (C_{20}-C_{36})) and the other half was analyzed for PAHs by gas chromatography coupled with mass spectrometry (GC/MS). Surrogate compounds were used to monitor extraction efficiency. Results were corrected based on respective surrogate recoveries.

Table 2. PAH analytes and abbreviations.

PAH	Abbreviation	PAH	Abbreviation
Biphenyl	B	Chrysene	CHR
Naphthalene	N	Chrysenes, C1 -	C1-CHR
Naphthalenes, C1 -	C1-N	Chrysenes, C2 -	C2-CHR
Naphthalenes, C2 -	C2-N	Chrysenes, C3 -	C3-CHR
Naphthalenes, C3 -	C3-N	Chrysenes, C4-	C4-CHR
Naphthalenes, C4 -	C4-N	Fluoranthene	FLA
Acenaphthylene	AY	Fluoranthene/Pyrenes, C1 -	C1-FLA
Acenaphthene	AE	Fluoranthene/Pyrenes, C2 -	C2-FLA
Anthracene	A	Fluoranthene/Pyrenes, C3-	C3-FLA
Dibenzothiophene	DBT	Fluoranthene/Pyrenes, C4 -	C3-FLA
Dibenzothiophenes, C1 -	C1-DBT	Pyrene	PY
Dibenzothiophenes, C2 -	C2-DBT	Benz[a]anthracene	BAA
Dibenzothiophenes, C3 -	C3-DBT	Benzo(b)fluoranthene	BBF
Fluorene	F	Benzo(k)fluoranthene	BKF
Fluorenes, C1 -	C1-F	Benzo(e)pyrene	BEP
Fluorenes, C2 -	C2-F	Benzo(a)pyrene	BAP
Fluorenes, C3 -	C3-F	Perylene	PER
Phenanthrene	P	Indeno(1,2,3-c,d) pyrene	IND
Phenanthrene/Anthracene, C1 -	C1-P	Dibenz(a,h)anthracene	DBA
Phenanthrene/Anthracene, C2 -	C2-P	Dibenz(a,h)anthracene, C1-	C1-DBA
Phenanthrene/Anthracene, C3 -	C3-P	Dibenz(a,h)anthracene, C2-	C2-DBA
Phenanthrene/Anthracene, C4 -	C4-P	Dibenz(a,h)anthracene, C3-	C3-DBA
		Benzo(g,h,i)perylene	BPER

Table 3. PAH metabolites and abbreviations.

Analyte Name	Abbreviation
4, 4'-dihydroxybiphenyl	di-OH-B
1-hydroxynaphthalene	1-OH-N
2-hydroxynaphthalene	2-OH-N
1- methyl-2hydroxynaphthalene + 6-methyl-2-hydroxynaphthalene	1+6-Me-2OH-N
6-methyl-2-hydroxynaphthalene	6Me-2OH-N
Methylated hydroxynaphthalenes	C1-OH-N
C2- dimethyl- and ethyl- hydroxynaphthalenes	C2-OH-N
Trimethyl-, methylethyl-, and propyl- hydroxynaphthalenes	C3-OH-N
Tetramethyl-, dimethylethyl-, diethyl-, methylpropyl- and butyl- hydroxynaphthalenes	C4-OH-N
Dihydroxydihydroanthracene with undetermined position	di-OH-A
1,8- *bis*(hydroxymethyl)anthracene	OH-Me-A
2-hydroxy-9,10-anthraquinone	2-OH-ATQ
1,5-dihydroxy- 1,2-dihydro-9,10-anthraquinone	di-OH-ATQ
2- hydroxydibenzothiophene	2-OH-DBT
Methylated hydroxydibenzothiophenes	C1-OH-DBT
Dimethyl- and ethyl- hydroxydibenzothiophenes	C2-OH-DBT
Trimethyl-, methylethyl-, and propyl- hydroxydibenzothiophenes	C3-OH-DBT
Tetramethyl-, dimethylethyl-, diethyl-, methylpropyl- and butyl- hydroxydibenzothiophenes	C4-OH-DBT
2-hydroxyfluorene	2-OH-F
3 hydroxyfluorene	3-OH-F
Methylated hydroxyfluorenes	C1-OH-F
Dimethyl- and ethyl- hydroxyfluorenes	C2-OH-F
Trimethyl-, methylethyl-, and propyl- hydroxyfluorenes	C3-OH-F
Tetramethyl-, dimethylethyl-, diethyl-, methylpropyl- and butyl- hydroxyfluorenes	C4-OH-F
1- hydroxyphenanthrene	1-OH-P
3-hydroxyphenanthrene + 2-hydroxyphenanthrene	3-OH-P + 2-OH-P
4-hydroxyphenanthrene	4-OH-P
9-hydroxyphenanthrene	9-OH-P
Methylated hydroxyphenanthrenes	C1-OH-P

Dimethyl- and ethyl- hydroxyphenanthrenes	C2-OH-P
1,2-dihydroxy-1,2- dihydrophenanthrene	1,2di-OH-P
9,10-dihydroxy-9,10- dihydrophenanthrene	9,10-di-OH-P
Dihydroxydihydrophenanthrene with undetermined position	di-OH-P
Methylated dihydroxydihydrophenanthrenes	C1-di-OH-P
Dimethyl- and ethyl- dihydroxydihydrophenanthrenes	C2-di-OH-P
Trimethyl-, methylethyl-, and propyl-dihydroxydihydrophenanthrenes	C3-di-OH-P
Tetramethyl-, dimethylethyl-, diethyl-, methylpropyl- and butyl- dihydroxydihydrophenanthrenes	C4-di-OH-P
cis-5,6-dihydroxy-5,6- dihydrobenz[*a*]anthracene	5,6-di-OH-BA
trans-8,9-dihydroxy-8,9- dihydrobenz[*a*]anthracene + *trans*-10,11-dihydroxy-10,11- dihydrobenz[a]anthracene	8,9 + 9,10-di-OH-BA
Dihydroxydihydrobenz[*a*]anthracene with undetermined	di-OH-BA
trans-1,2-dihydroxy-1,2- dihydrochrysene	1,2-di-OH-CHR
trans-3,4-dihydroxy-3,4- dihydrochrysene	3,4-di-OH-CHR
trans-5,6-dihydroxy-5,6- dihydrochrysene	5,6-di-OH-CHR
trans-2,3-dihydroxy- 2,3-dihydrofluoranthene	2,2-di-OH-FLA
Methylated dihydroxydihydrofluoranthenes	C1-di-OH-FLA
Dimethyl- and ethyl- dihydroxydihydrofluoranthenes	C2-di-OH-FLA
Trimethyl-, methylethyl-, and propyl-dihydroxydihydrofluoranthenes	C3-di-OH-FLA
Tetramethyl-, dimethylethyl-, diethyl-, methylpropyl- and butyl- dihydroxydihydrochrysenes	C4-di-OH-FLA
cis-5,6-dihydroxy-5,6- dihydrobenzo[*a*]pyrene	4,5-di-OH-BAP
cis-7,8-dihydroxy-7,8- dihydrobenzo[*a*]pyrene	7,8-di-OH-BAP
Dihydroxydihydrobenzo[*a*]pyrene with undetermined position	di-OH-BAP
r7,t8,t9,c10-tetrahydroxy-7,8,9,10-tetrahydrobenzo[a]pyrene	7,8,9,10-tetra-OH-BAP
trans-4,5-dihydroxy-4,5- dihydrobenzo[*e*]pyrene	4,5-di-OH-BEP

Results

Test 1

No mortality or abnormal behavior was observed in either the treatment group or control, during the static exposure to the 1 g/L HEWAF. Fish mean fork length was 11.4 cm and their mean body weight was 10.6 grams. Water quality was within acceptable parameters.

Water chemistry results showed a significant decrease over the 72-hour exposure period in both TPH and PAH concentrations. The TPH diesel range organics (C_{10}-C_{28}) water concentration declined 65% over the exposure period, but the motor oil range (C_{20}-C_{36}) loss was only 12% (Table 4). In terms of PAH loss, naphthalenes showed the greatest loss over time (Figure 1).

Table 4. TPH HEWAF concentrations at Test 1 start (Day 0) and termination (Day 3), including percent loss over the exposure period.

Parameter	Day 0 (mg/L)	Day 3 (mg/L)	% Loss
TPH (C_{10}-C_{28})	24.5	8.48	65%
TPH (C_{20}-C_{36})	20.6	18.1	12%
TPH Sum	45.1	26.6	41%

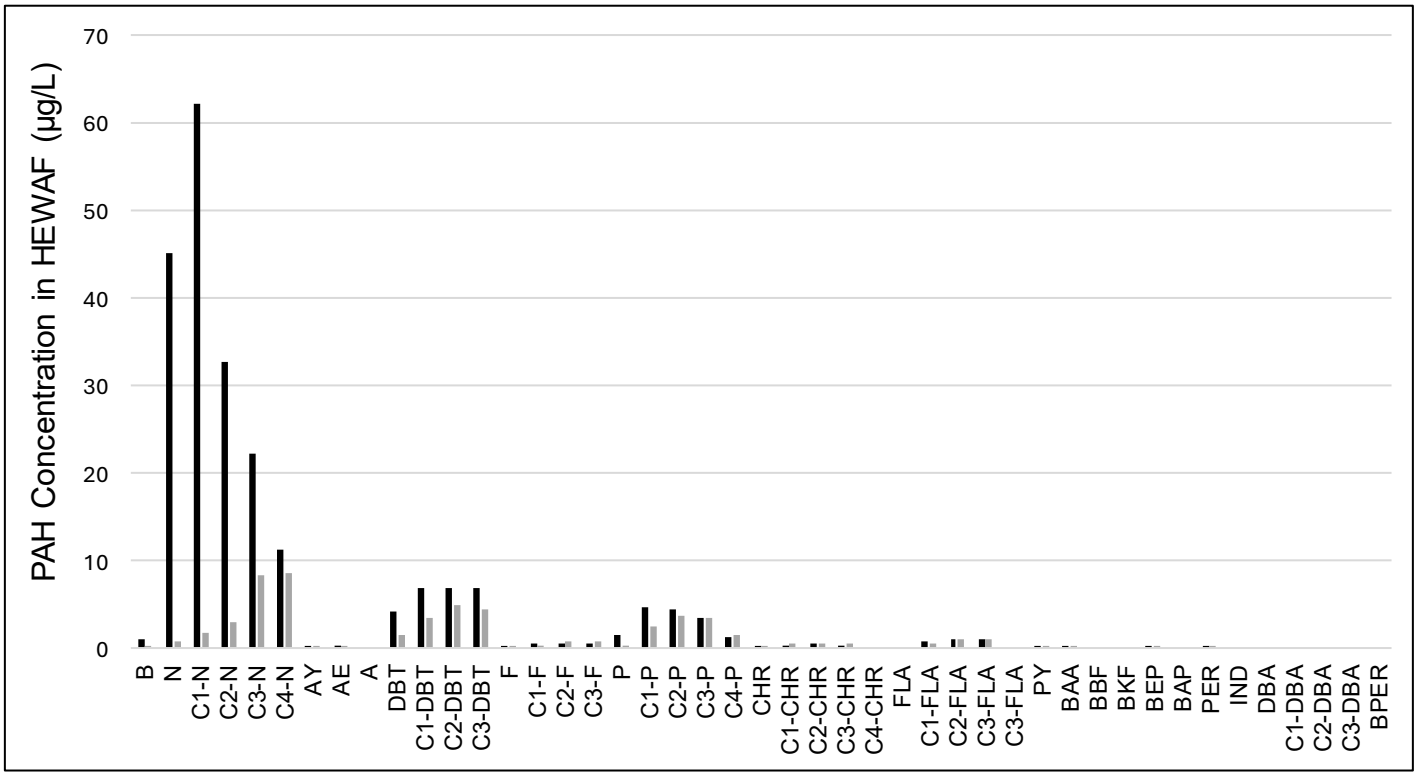

Figure 1. Test 1 PAH concentrations in HEWAF on Day 0 (■) and Day 3 (■).

Compared to the Day 0 seawater control, the Day 0 HEWAF had a brownish color but by Day 3, it was clear with some oil droplets visible on the water's surface and sides of the tank (Figure 2). The visual decrease of oil in the water column over the exposure period is supported by the water chemistry data.

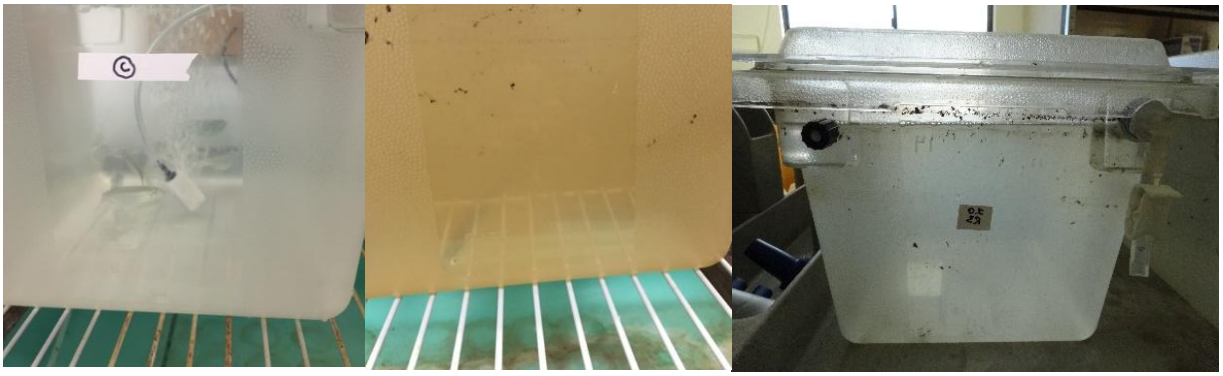

Figure 2. Test 1 Day 0 seawater control (left), Day 0 HEWAF (center), and Day 3 HEWAF (right).

Evidence of oil uptake was confirmed by the biliary PAH metabolite profiles of the exposed fish (Figure 3). The most abundant PAH metabolites detected were methylated hydroxydibenzothiophenes (C1-hydroxyDBT, C2-hydroxyDBT and C4-hydroxyDBT) Other abundant metabolites included phenanthrenes, fluorenes, and fluoranthenes. Despite parent and alkylated naphthalene having the greatest concentration in the HEWAF at the start of the experiment relative to the other PAHs measured, the metabolites of these compounds were relatively lower in the bile compared to dibenzothiophenes, fluorenes, some phenanthrenes and some fluoranthenes.

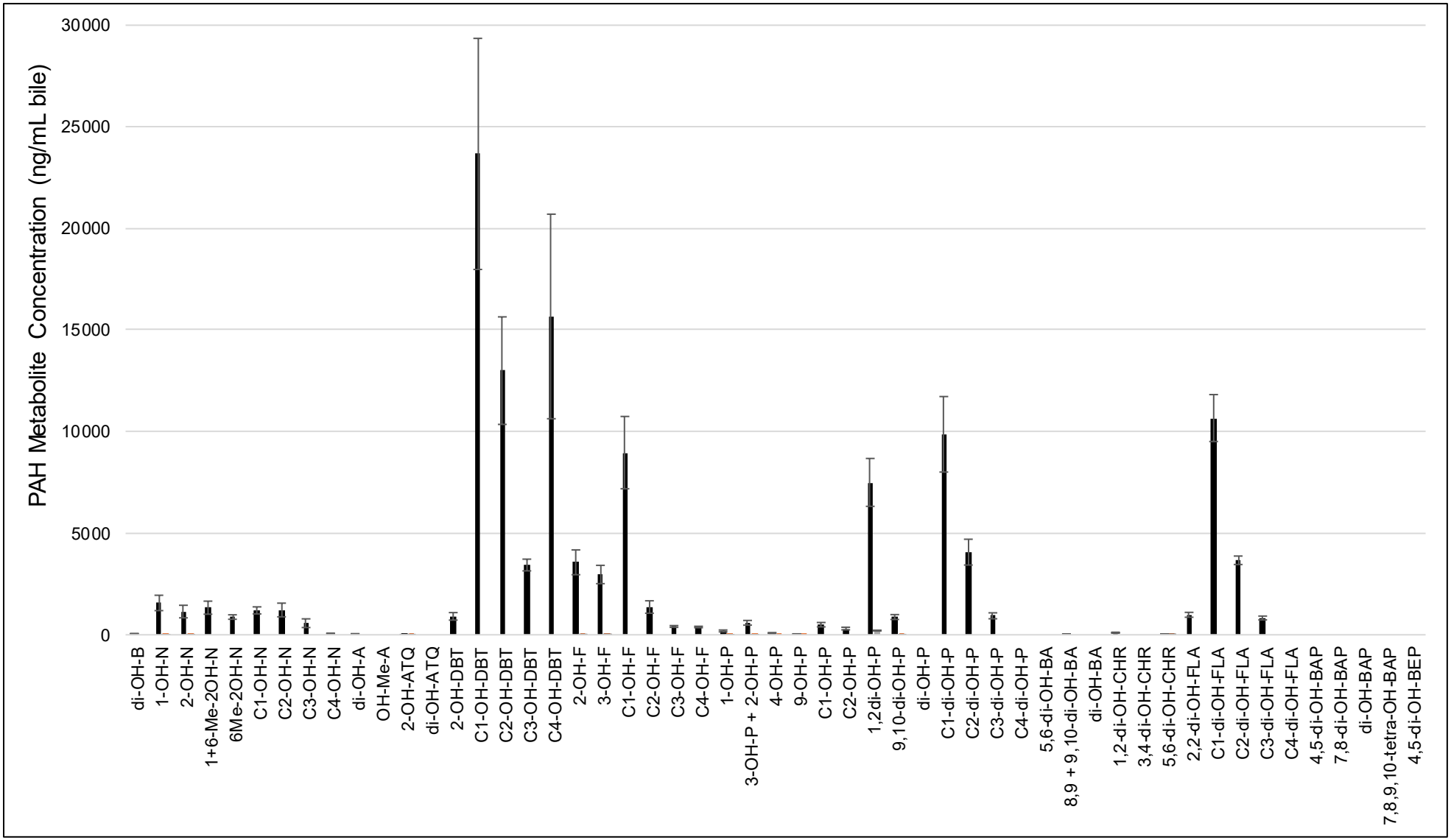

Figure 3. Average treatment (■) biliary PAH metabolite concentrations (ng/mL) following 72-hour static exposure to 1 g/L HEWAF in Test 1 as measured by the NOAA laboratory. Error bars represent the standard deviation. PAH metabolite concentrations measured in control fish are not represented due to low measured levels.

Test 2

Mortality resulted only in the unweathered, daily renewal treatment (Table 5). Fish mean fork length was 11.3 cm and their mean body weight was 10.0 grams. Water quality was within acceptable parameters. Dark red areas were observed around the snout of 93% of the fish in the unweathered oil treatment (Figure 4) and these fish were observed swimming at the surface more often than the other treatments. Caudal fin erosion was also noted in this treatment group.

Table 5. Daily and cumulative mortality results (#dead/total # fish) for Test 2.

Treatment	24 hr.	48 hr.	72 hr.	% Mortality
Control Static	0/30	0/30	0/30	0
Unweathered Daily Renewal	0/30	1/30	16/29	56.7
Weathered Static	0/30	0/30	0/30	0

Figure 4. Example of red lesion observed around the snout of topsmelt in the unweathered oil treatment during Test 2.

In the unweathered HEWAF, TPH concentrations (Table 6) were slightly higher than the Test 1 bioassay (Table 4). On Day 0, TPH concentrations in the weathered HEWAF were lower than the unweathered HEWAF treatment (Table 6). A comparison of the PAH composition of the two HEWAFs, based on concentration, is shown in Figures 5 and 6. Both HEWAFs were dominated by naphthalenes.

In the weathered HEWAF treatment, there was a 65% loss in TPH (C_{10}-C_{28}) and an 11% loss in TPH (C_{20}-C_{36}) by Day 3 (Table 6). There was a 34% loss in TPH (C_{10}-C_{28}) but a 29% increase in TPH (C_{20}-C_{36}) by Day 3 in the unweathered HEWAF with daily renewal. A comparison of the PAH concentrations on Day 0 and Day 3 in the weathered and unweathered HEWAF showed loss of naphthalenes over time (Figures 5 and 6).

Daily renewals showed to be important for maintaining a more consistent exposure concentration, in addition to lack of tank aeration.

Table 6. TPH HEWAF concentrations for weathered HEWAF (no renewal) and unweathered HEWAF (daily renewal) during Test 2, including percent loss over the exposure period.

Parameter	Day 0 Unweathered HEWAF (mg/L)	Day 3 Unweathered HEWAF (mg/L)	% Loss	Day 0 Weathered HEWAF (mg/L)	Day 3 Weathered HEWAF (mg/L)	% Loss
TPH (C_{10}-C_{28})	35.8	23.7	34%	5.27	1.86	65%
TPH (C_{20}-C_{36})	28.5	36.7	-29%	4.03	3.60	11%
TPH Sum	64.3	60.4	6%	9.30	5.46	41%

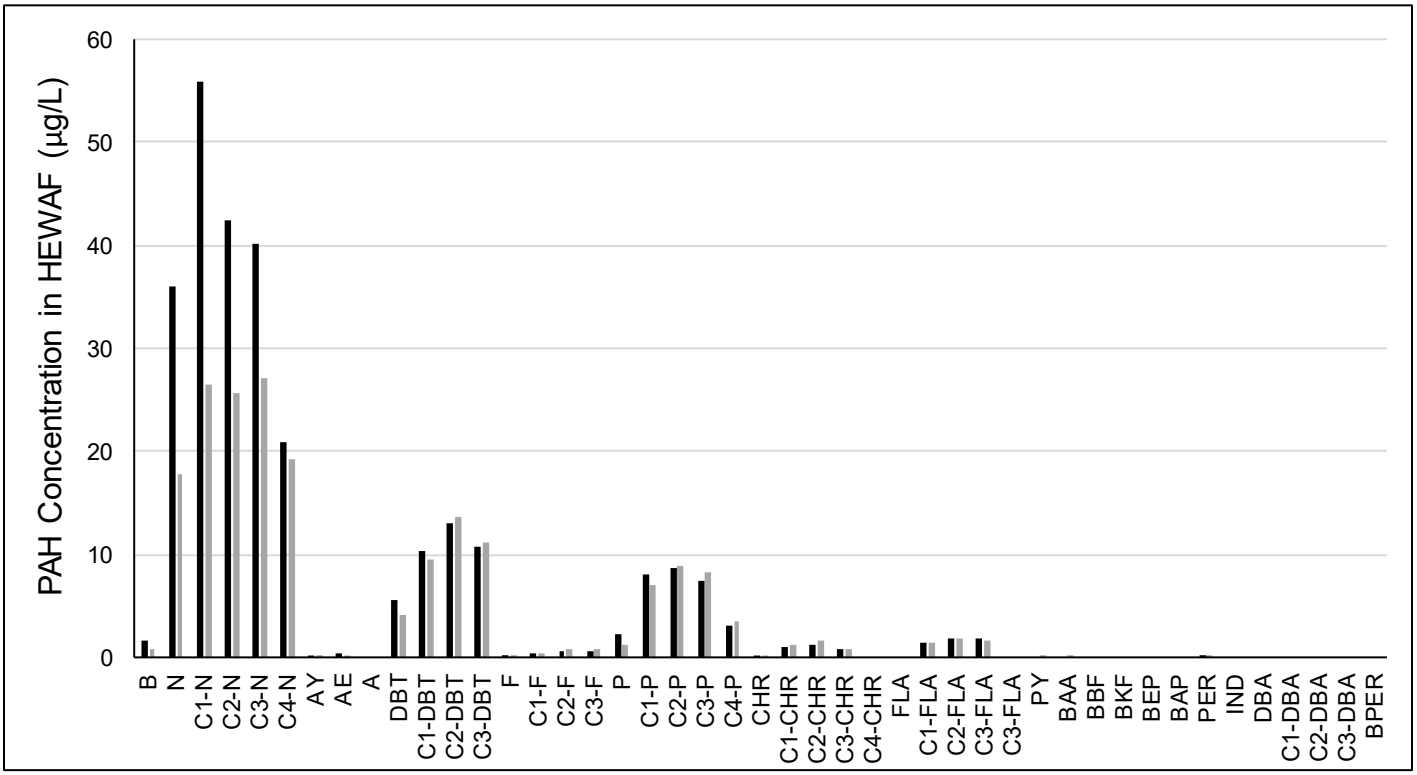

Figure 5. PAH concentrations in unweathered HEWAF (daily renewal) for Test 2 start (Day 0 ■) and finish (Day 3 ■).

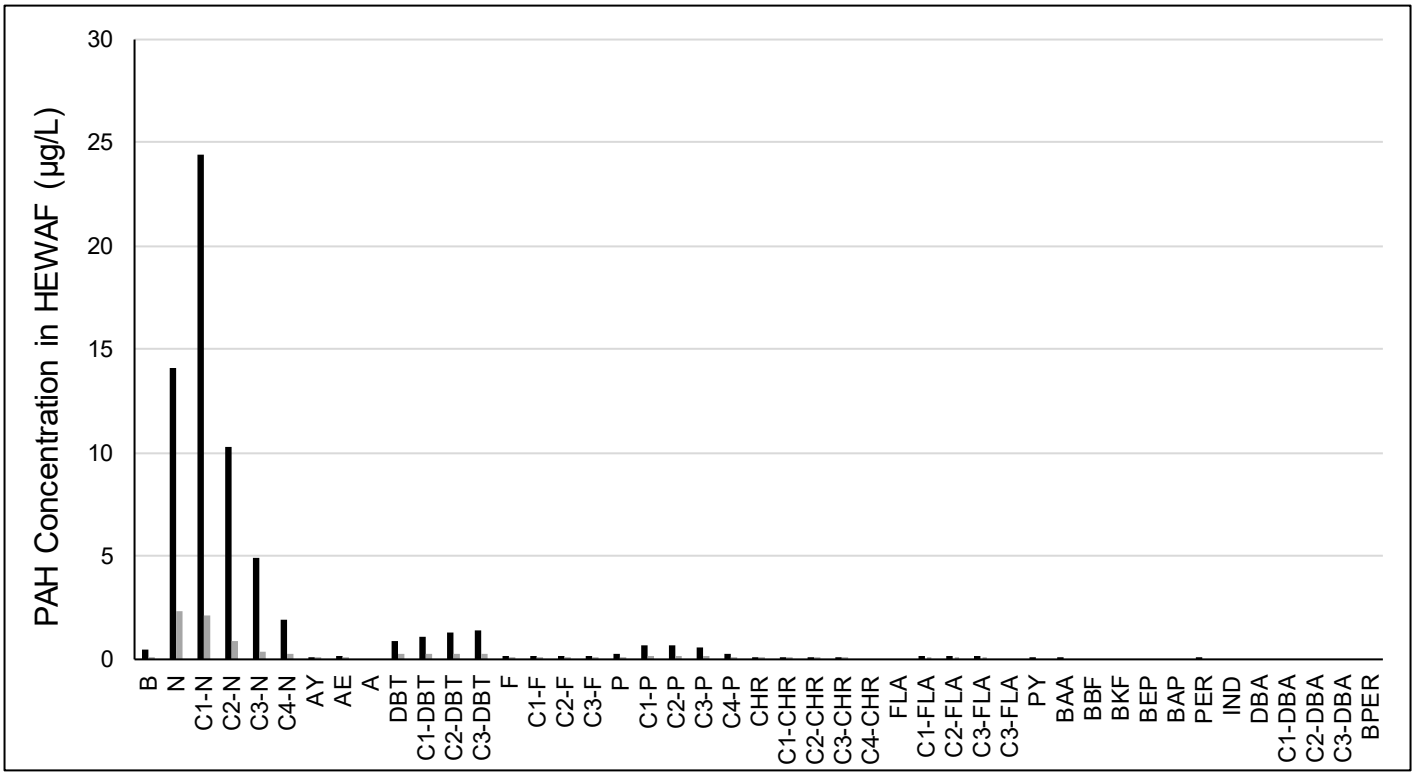

Figure 6. PAH concentrations in weathered HEWAF (no renewal) for Test 2 start (Day 0 ■) and finish (Day 3 ■).

Evidence of oil uptake was confirmed by the biliary PAH metabolite profiles of the exposed fish (Figure 7). The first two replicates from the unweathered treatment consist of bile from live fish whereas the bile from replicate three was collected from dead fish. As a result, replicate three for the unweathered treatment was not included in the average calculation.

In general, metabolite concentrations were greater with daily HEWAF renewal of unweathered oil compared to without renewal (Figure 7). In the unweathered treatment with daily renewal, the most abundant metabolites were methylated hydroxydibenzothiophenes (C1-OH-DBT, C2-OH-DBT and C4-OH-DBT), methylated dihydroxydihydrofluoranthenes (C1-di-OH-FLA), methylated hydroxyfluorenes (C1-OH-F), and methylated dihydroxydihydrophenanthrenes (C1-di-OH-P and 1,2di-OH-P), respectively. As seen previously, despite the parent and alkylated naphthalenes having the greatest concentrations in HEWAF relative to other PAHs measured, the metabolites of these compounds were not dominant in the bile profile. In the weathered treatment without daily renewal, the most abundant metabolites were methylated dihydroxydihydrofluoranthenes (C1-di-OH-FLA) followed by methylated hydroxydibenzothiophenes (C1-OH-DBT, C2-OH-DBT and C4-OH-DBT), dimethyl- and ethyl- dihydroxydihydrofluoranthenes (C2-di-OH-FLA), methylated dihydroxydihydrophenanthrenes (C1-di-OH-P), and 1,2-dihydroxy-1,2-dihydrophenanthrene (1,2di-OH-P), respectively.

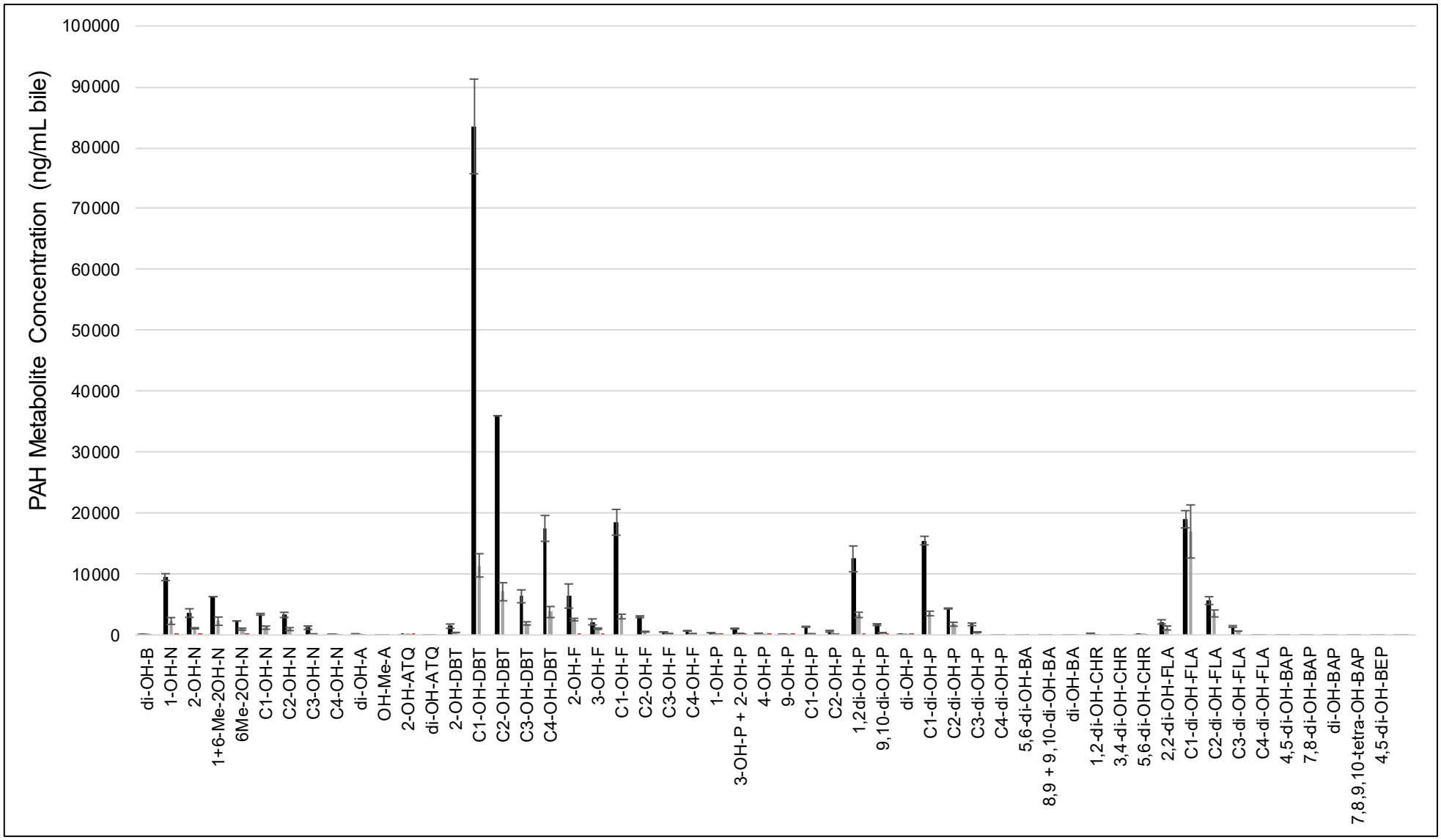

Figure 7. Average biliary PAH metabolites (ng/mL) following 72-hour exposure to 1 g/L unweathered HEWAF(■) and 1 g/L weathered HEWAF (■) in Test 2 as measured by the NOAA laboratory. The average value for the unweathered treatment only consists of 2 replicates since the bile from replicate #3 was from dead fish. Error bars represent the standard deviation.

Over the 72-hr. exposure, fish behavior observations were recorded daily. On Day 1, fish in each treatment displayed normal swimming and normal social interaction. Control and weathered HEWAF exposed fish preferred to rest on the bottom of their tanks, whereas unweathered HEWAF exposed fish preferred floating at the surface of the tank. Changes in snout color in the unweathered treatment were observed at this time. On Day 2, swimming, social interaction and tank location observations remained the same as the day before, however labored breathing was observed in unweathered HEWAF exposed fish. At test termination, swimming and tank location were not different between the controls and weathered HEWAF treatments. However, in the unweathered HEWAF treatment, surviving fish in the second replicate displayed loss of equilibrium and corkscrew swimming. In addition, these fish were breathing heavier.

At test termination, fish were recorded as replicate groups and individually to identify changes in their behavior. When placed into a secondary tank for recording, some fish from the control and weathered treatments displayed erratic swimming, constant swimming or perpendicular orientation during the 5-minute recording. Fish in the unweathered HEWAF treatment did not display those changes in swimming. During the 5-minute recording, stimuli in the form of sound was introduced to determine whether exposed fish responded differently than control fish. Stimuli were applied to replicate groups and individuals. Fish responses were scored and totaled for each treatment (Table 7). Responses from fish varied between the treatments. Control fish responded more frequently to the applied stimuli whereas fish in the unweathered HEWAF treatment responded more frequently than the weathered treatment; however, there were less fish in the unweathered treatment due to mortality. When fish were recorded in a group, the responses were variable. The controls were less responsive than the weathered HEWAF treatment. The unweathered HEWAF groups were less responsive than the controls and weathered treatment.

Table 7. Summary of stimuli responses from Test 2. Results are displayed as ratios of elicited response out of the total number of responses possible. For the group, responses are the total number of fish per stimuli that elicited a response out of the total number of responses possible.

Treatment	Replicate A	Replicate B	Replicate C	Replicate Overall	Group Overall
Control Static	17 / 35	28 / 35	17 / 35	62 / 105 (59%)	47 / 105 (45%)
Unweathered Daily Renewal	17 / 35	11 / 28		28 / 63 (44%)	24 / 63 (38%)
Weathered Static	12 / 35	14 / 35	14 / 35	40 / 105 (38%)	62 / 105 (59%)

Segments of the 5-minute recordings, that contained the individual fish and the stimuli only (e.g., 36.3 seconds), were processed with the Animal Tracker plugin loaded into the Image J software. The Animal Tracker plugin measures the speed (e.g., velocity) and distance travelled by the fish during the recorded segment. Each individual fish video was processed, and the overall mean velocity (Figure 8) and mean distance (Figure 9) were determined for each oil treatment and control. Results show that fish exposed to unweathered oil moved slower and not as much as the control fish or those exposed to weathered oil. This indicates that fish exposed to unweathered oil were experiencing narcosis. Thus, exposure to 1 g/L unweathered HEWAF resulted in reduced response to stimuli.

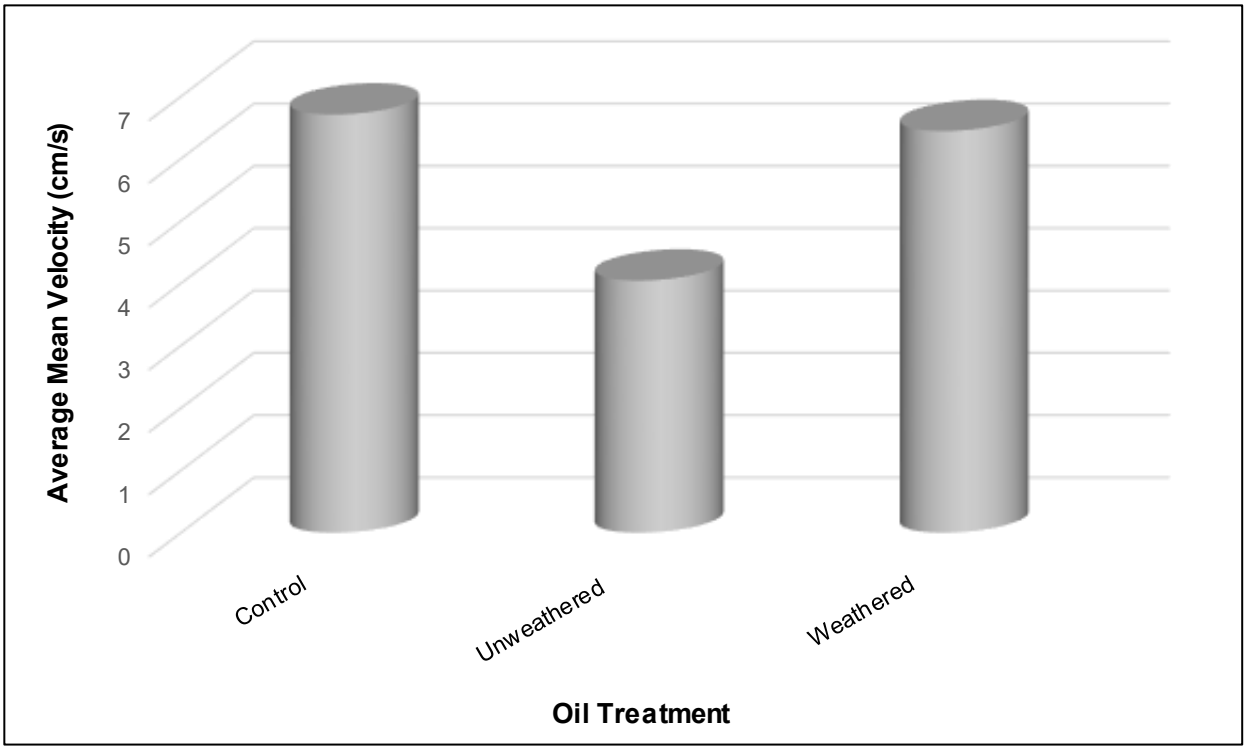

Figure 8. Average mean velocity of replicate fish per oil treatment (control, unweathered or weathered) in Test 2.

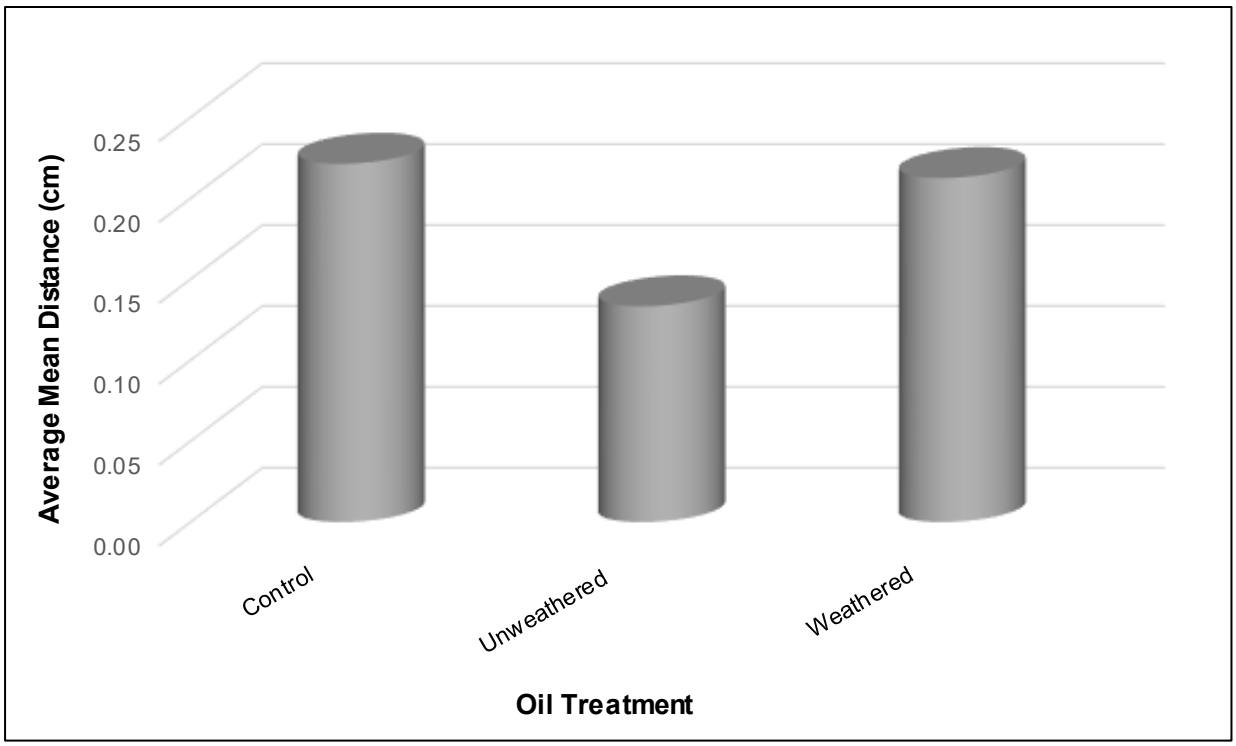

Figure 9. Average mean distance of replicate fish per oil treatment (control, unweathered or weathered) in Test 2.

Test 3

Mortality in the control tanks resulted from two fish escaping the tanks and four fish dying for unknown reasons. Although mass mortality did not occur in the oil treatments, the 0.5 g/L treatment did have recorded mortality from one fish escaping one tank and another fish dying for unknown reasons (Table 8). Fish mean fork length was 13.2 cm and their mean body weight was 20.3 grams. Water quality was within acceptable parameters. Unlike Test 2, fish exposed to the unweathered HEWAF at 1 g/L did not display red areas around their snouts. It should be noted that the fish used in Test 3 were twice the weight of the fish used in Tests 1 and 2.

Table 8. Daily and cumulative mortality results (#dead/total # fish) for Test 3.

Treatment	24 hr.	48 hr.	72 hr.	% Mortality
Control Static	2/24	0/22	4/22	25%
0.5 g/L Unweathered Daily Renewal	0/24	1/24	1/23	8%
1.0 g/L Unweathered Daily Renewal	0/24	0/24	0/24	0%

The percentage loss of TPH concentrations are presented in Table 9. Over the course of the experiment, despite daily renewals, 65% of the TPH (C_{10}-C_{28}) concentration was lost in the 0.5 g/L loading rate and 69% (C_{10}-C_{28}) was lost in the 1.0 g/L loading rate. The diesel range hydrocarbons, which have smaller molecular weights, had an increased loss compared to the larger, motor oil range hydrocarbons.

The HEWAF PAH profiles were dominated by naphthalenes in both the 0.5 g/L and 1.0 g/L loading rates (Figure 10). The PAH distribution, on a concentration basis, is presented in Figures 10 and 11.

Table 9. TPH HEWAF concentrations at 0.5 g/L and 1.0 g/L loading rates during Test 3, including percent loss over the exposure period.

Parameter	Day 0 0.5 g/L HEWAF (mg/L)	Day 3 0.5 g/L HEWAF (mg/L)	% Loss	Day 0 1.0 g/L HEWAF (mg/L)	Day 3 1.0 g/L HEWAF (mg/L)	% Loss
TPH (C_{10}-C_{28})	4.85	1.68	65%	12.8	4.02	69%
TPH (C_{20}-C_{36})	5.05	5.22	-3%	12.2	13.7	-12%
TPH Sum	9.9	6.9	30%	25	17.72	29%

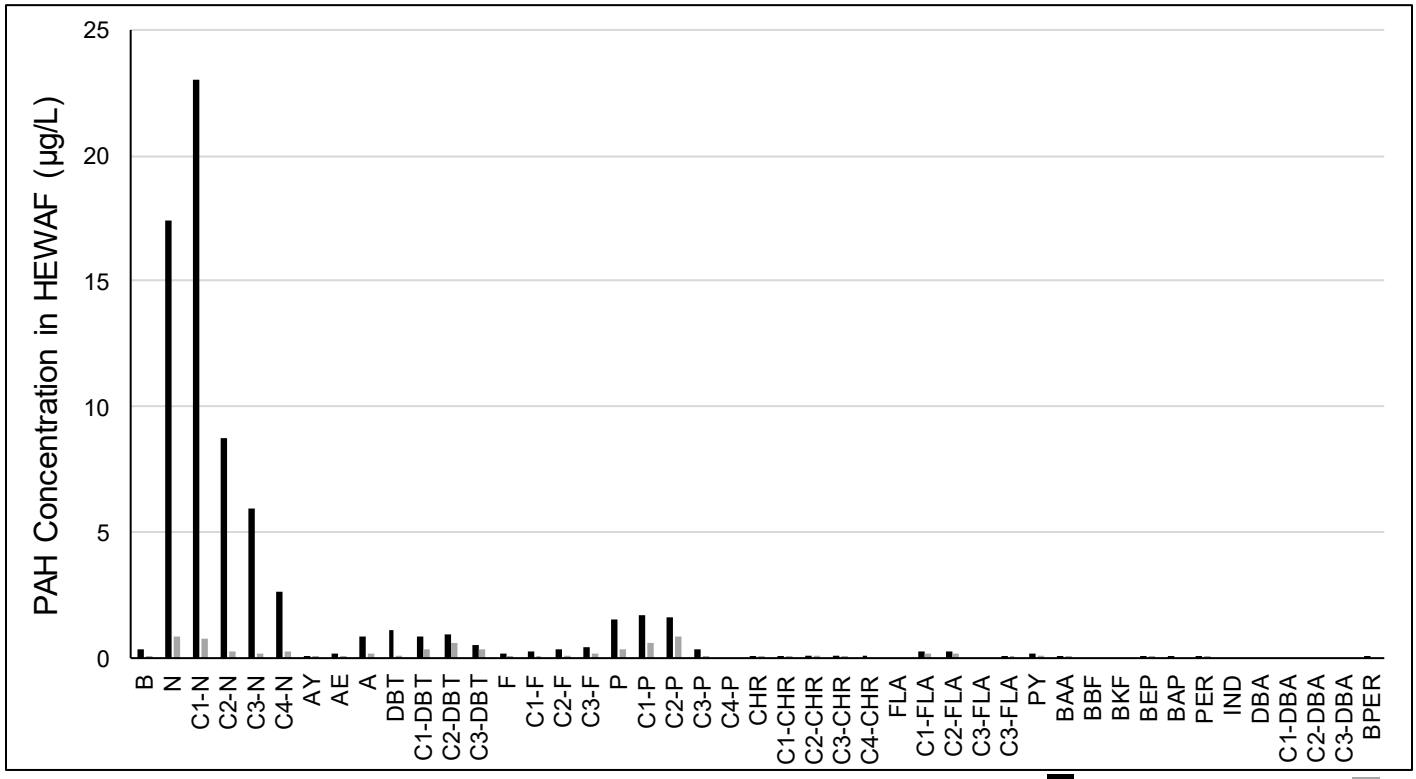

Figure 10. PAH concentrations in 0.5 g/L HEWAF for Test 3 start (Day 0 ■) and finish (Day 3 ■).

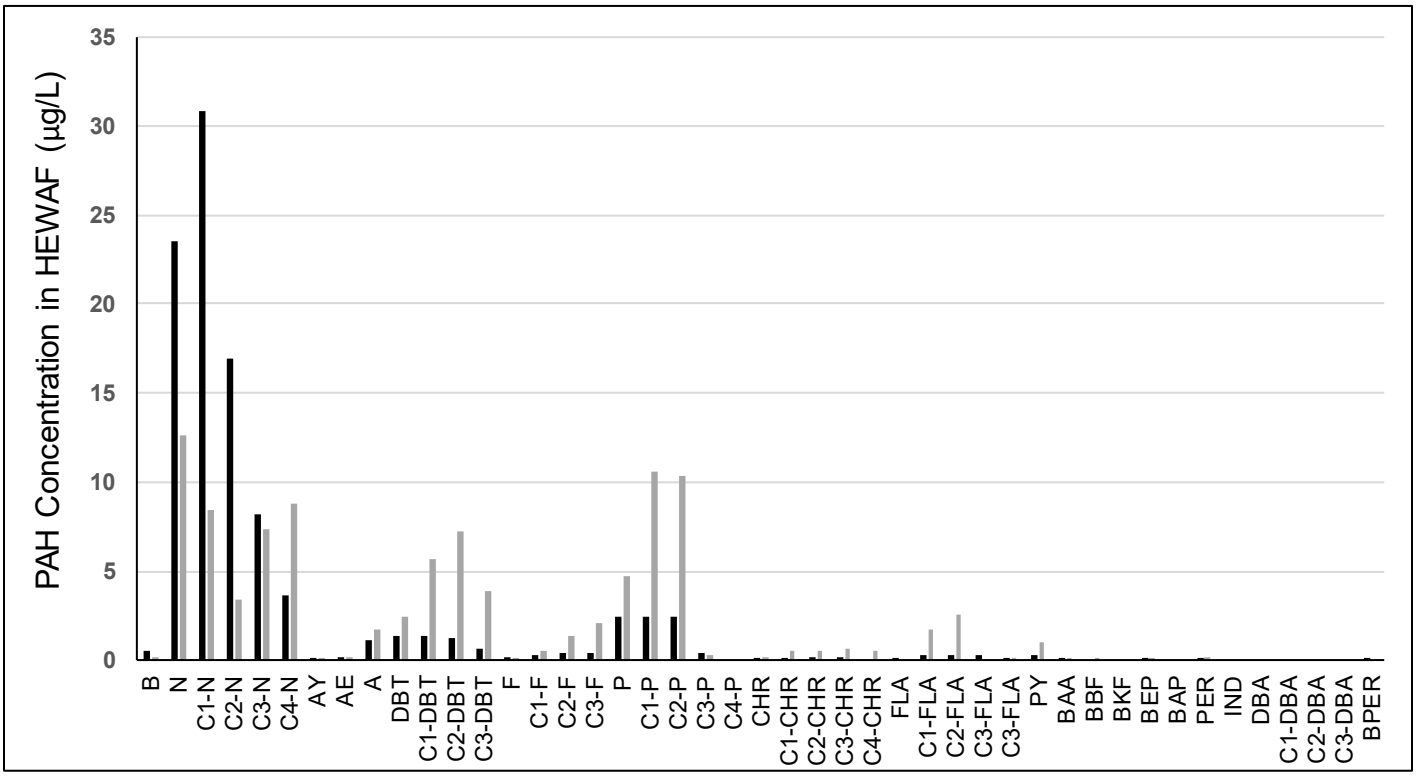

Figure 11. PAH concentrations in 1.0 g/L HEWAF for Test 3 start (Day 0 ■) and finish (Day 3 ■).

The biliary PAH metabolite profiles for both loading rates are shown in Figure 12. Metabolite concentrations increased with increasing loading rate, although not as a linear response. In the 0.5 g/L loading rate, the most abundant metabolites were methylated dihydroxydihydrofluoranthenes (C1-di-OH-FLA), hydroxydibenzothiophenes (C1-OH-DBT and C2-OH-DBT), methylated hydroxyfluorenes (C1-OH-F), dihydroxydihydrophenanthrenes (di-OH-P and C2-di-OH-P), and methylated hydroxyphenanthrenes (C1-OH-P), respectively.

In the 1.0 g/L loading rate, the most abundant metabolites were hydroxydibenzothiophenes (C1-OH-DBT, C2-OH-DBT)dihydroxydihydrofluoranthenes (C1-di-OH-FLA and C2-di-OH-FLA),), methylated hydroxyfluorenes (C1-OH-F), hydroxynaphthalenes (C1-OH-N and C2-OH-N), dihydroxydihydrophenanthrenes with undetermined position (di-OH-P), di and methylated hydroxyphenanthrene (C1-OH-P), , respectively.

As seen previously, despite the parent and alkylated naphthalenes having the greatest concentrations in HEWAF relative to other PAHs measured, the metabolites of these compounds were not dominant in the profile.

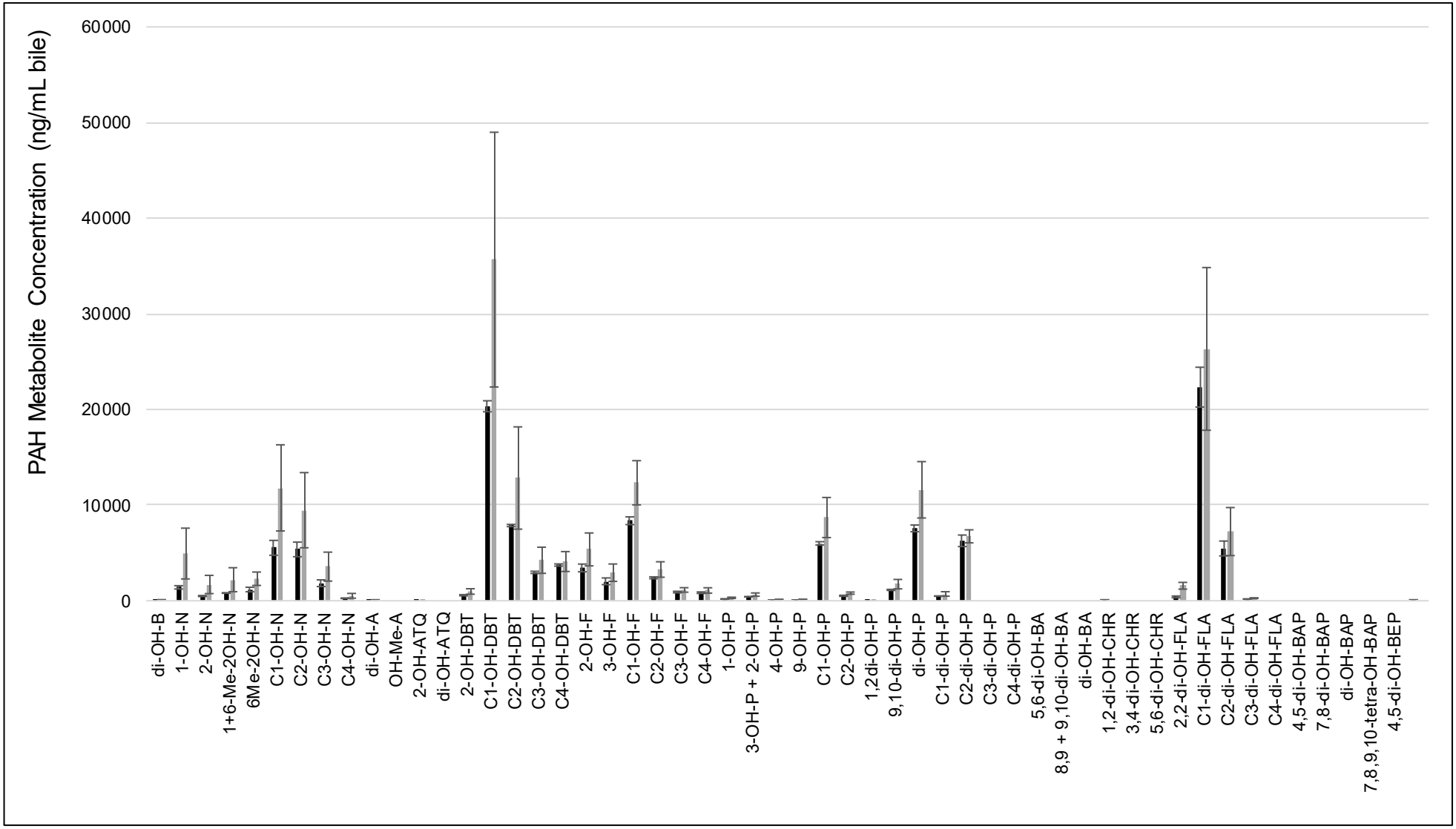

Figure 12. Average biliary PAH metabolites (ng/mL) following 72-hour exposure to 0.5 g/L HEWAF(■) and 1.0 g/L HEWAF (▒) in Test 3 as measured by the NOAA laboratory. Error bars represent the standard deviation.

Over the 72-hr. exposure, fish behavior observations were recorded daily. At test termination, swimming and tank location were not different between the controls and HEWAF treatments. In the 1 g/L HEWAF treatment, some surviving fish had loss of equilibrium and corkscrew swimming.

At test termination, fish were recorded as replicate groups and individually to identify changes in their behavior. When placed into a secondary tank for recording, some fish from the control and oil treatments displayed erratic swimming, constant swimming or perpendicular orientation during the 5-minute recording. During the 5-minute recording, stimuli in the form of sound was introduced in order to determine whether exposed fish responded differently than the control fish. Stimuli were applied to replicate groups and individuals. Fish responses were scored and totaled for each treatment (Table 10). Responses from fish varied between the treatments. Control and 0.5 g/L treated fish responded more frequently to the applied stimuli whereas, 1.0 g/L treated fish responded less frequently. When fish were recorded in a group of three or four fish, the responses were similar between the controls and the two treatments.

Table 10. Summary of stimuli responses for Test 3. Results are displayed as ratios of elicited response out of the total number of responses possible. For the group, responses are the total number of fish per stimuli that elicited a response out of the total number of responses possible.

Treatment	Replicate A	Replicate B	Replicate C	Replicate Overall	Group Overall
Control Static	9 / 21	15 / 21	10 /21	34 / 63 (54%)	39 / 60 (65%)
0.5 g/L Unweathered Daily Renewal	16 / 28	21 / 28	12 / 28	46 / 84 (55%)	45 / 84 (54%)
1.0 g/L Unweathered Daily Renewal	7 / 28	4 / 28	10 / 28	21 / 84 (25%)	55 / 84 (65%)

Segments of the 5-minute recordings, that contained the individual fish and the stimuli only (e.g., 36.3 seconds), were processed with the Animal Tracker plugin loaded into the *Image J* software. The Animal Tracker plugin measures the speed (e.g., velocity) and distance travelled by the fish during the recorded segment. Each individual fish video was processed, and the overall mean velocity (Figure 13) and mean distance (Figure 14) were determined for each oil treatment and control. Results show that fish exposed to the 1.0 g/L unweathered oil moved slower and not as much as the control fish or those exposed to 0.5 g/L unweathered oil. This indicates that fish exposed to 1.0 g/L unweathered oil were experiencing narcosis, thus, exposure resulted in reduced response to stimuli.

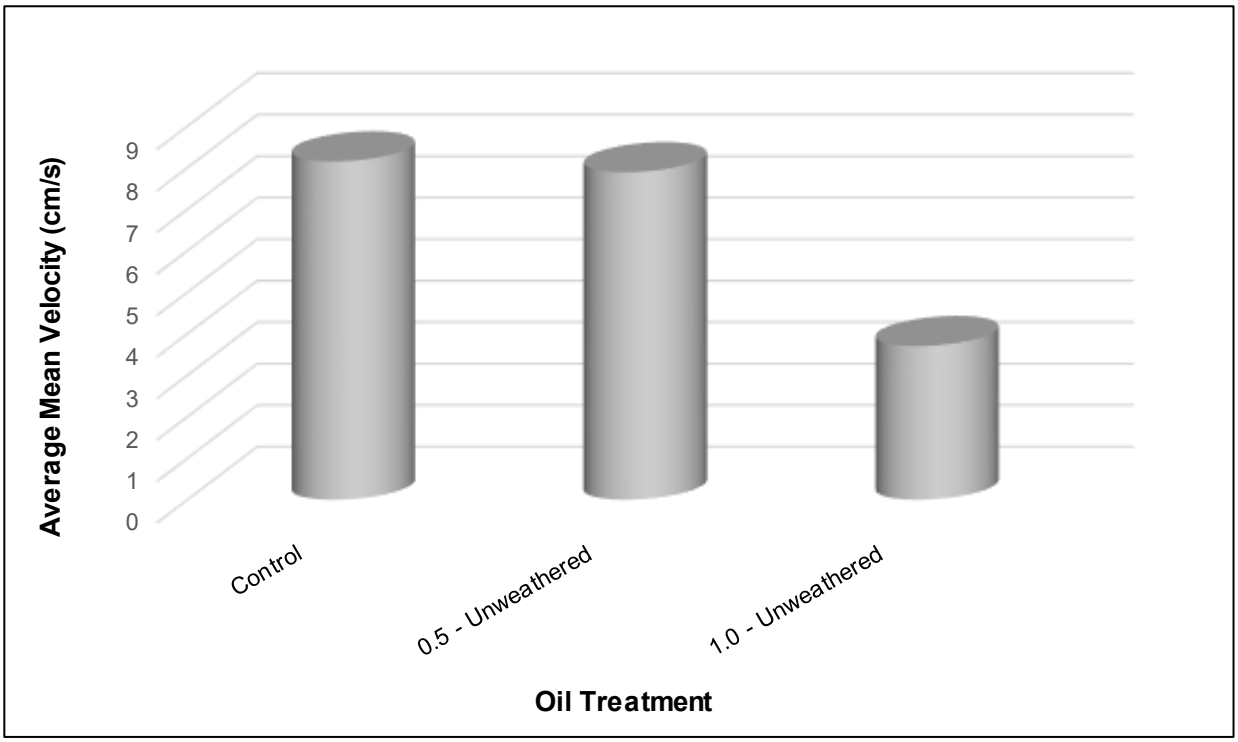

Figure 13. Average mean velocity of replicate fish per oil treatment (control, 0.5 g/L or 1.0 g/L) in Test 3.

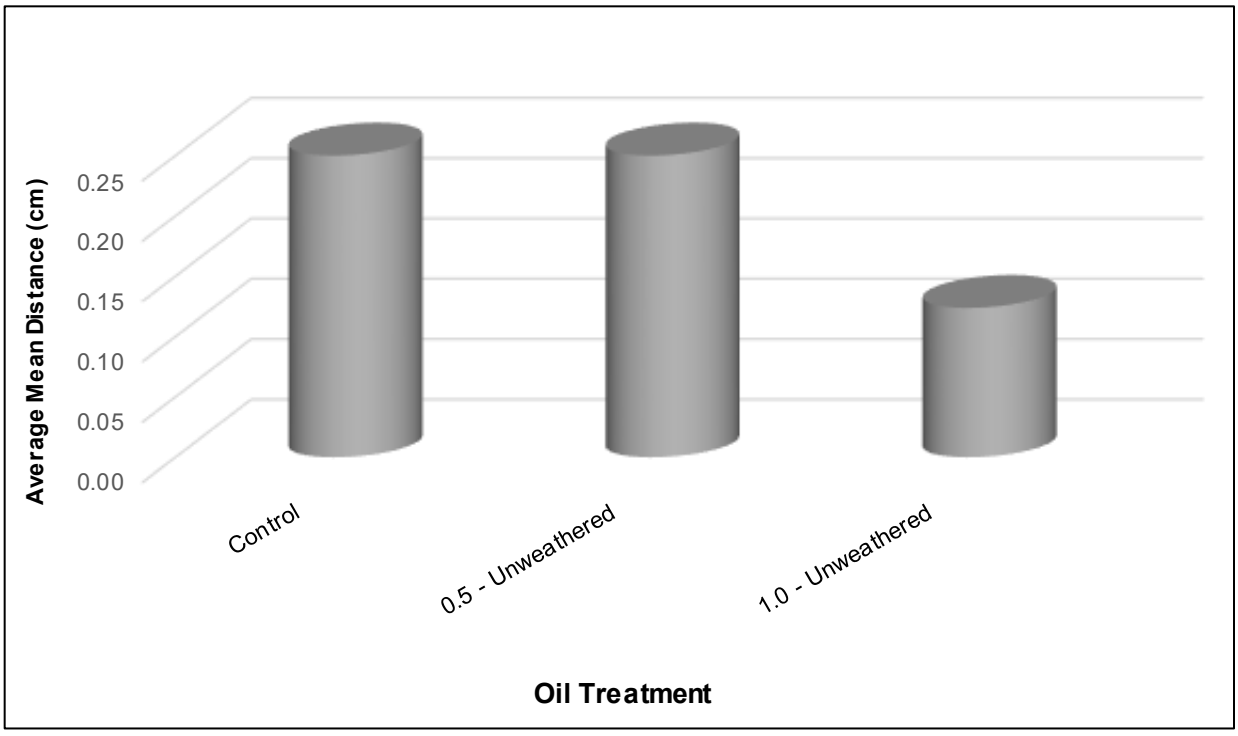

Figure 14. Average mean distance of replicate fish per oil treatment (control, 0.5 g/L or 1.0 g/L) in Test 3.

Interlaboratory Comparison

A comparison of the individual bile sample results for analytes in common to both laboratories are presented in Figure 15 for Test 1, Figure 16 for Test 2, and Figure 17 for Test 3. In HEWAF exposed samples, the relative percent difference (RPD) for common analytes ranged from 19% to 81% in Test 1, compared with 31% to 84% in Test 2, and 26% to 60% in Test 3. The RPD from control samples showed larger RPD values compared to the HEWAF exposed samples in all treatments as a result of greater variation at very low concentration levels.

In general, similar profiles of PAH metabolites were demonstrated in both laboratories. The NOAA method contains a much broader range of metabolites, including dihydroxylated and alkylated PAH metabolites allowing for a more in depth analysis of the bile samples.

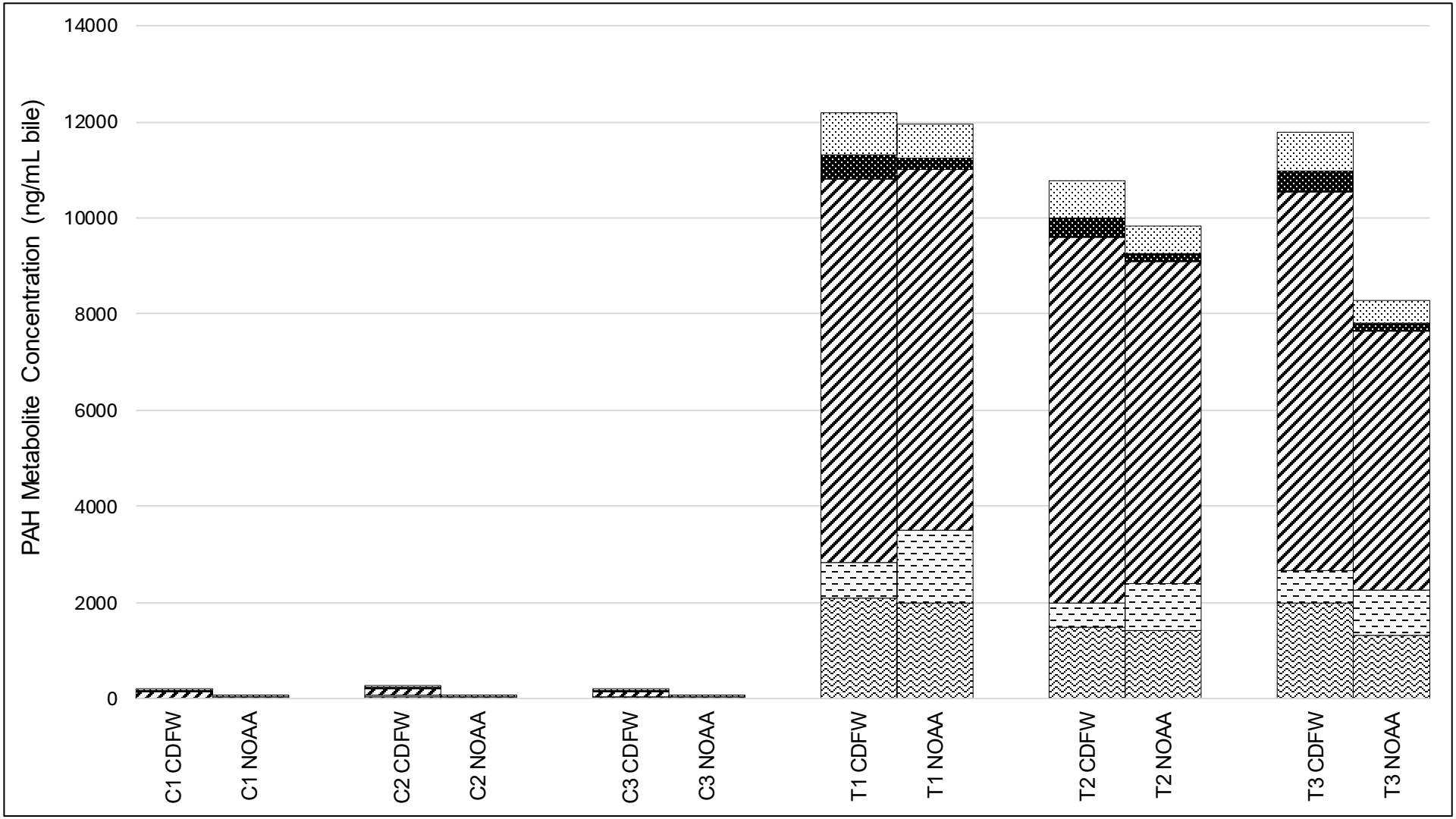

Figure 15. Test 1 replicate comparison of individual biliary PAH metabolite sample results, for overlapping target analytes between the NOAA and CDFW laboratories.
C = control, T = treatment. 1-OH-N ( ), 2-OH-N ( ), 2-OH-F/ 3-OH-F ( ), 2-OH-P/ 3-OH-P ( ), 1-OH-P ( ).

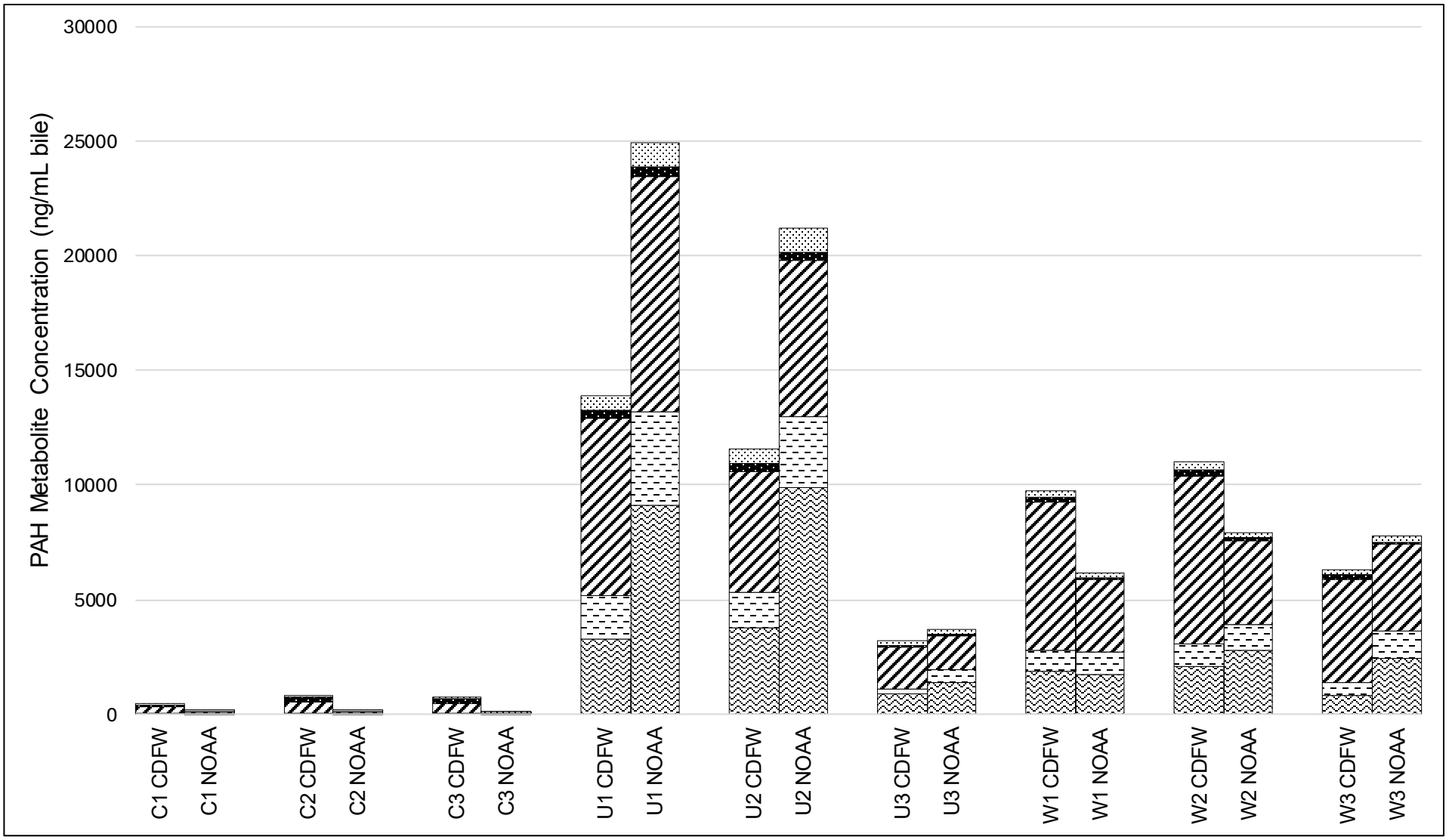

Figure 16. Test 2 replicate comparison of individual biliary PAH metabolite sample results for overlapping target analytes between the NOAA and CDFW laboratories.

C = control, U = unweathered HEWAF, W = weathered HEWAF. 1-OH-N ( ), 2-OH-N ( ), 2-OH-F/ 3-OH-F ( ), 2-OH-P/ 3-OH-P ( ), 1-OH-P ( ).

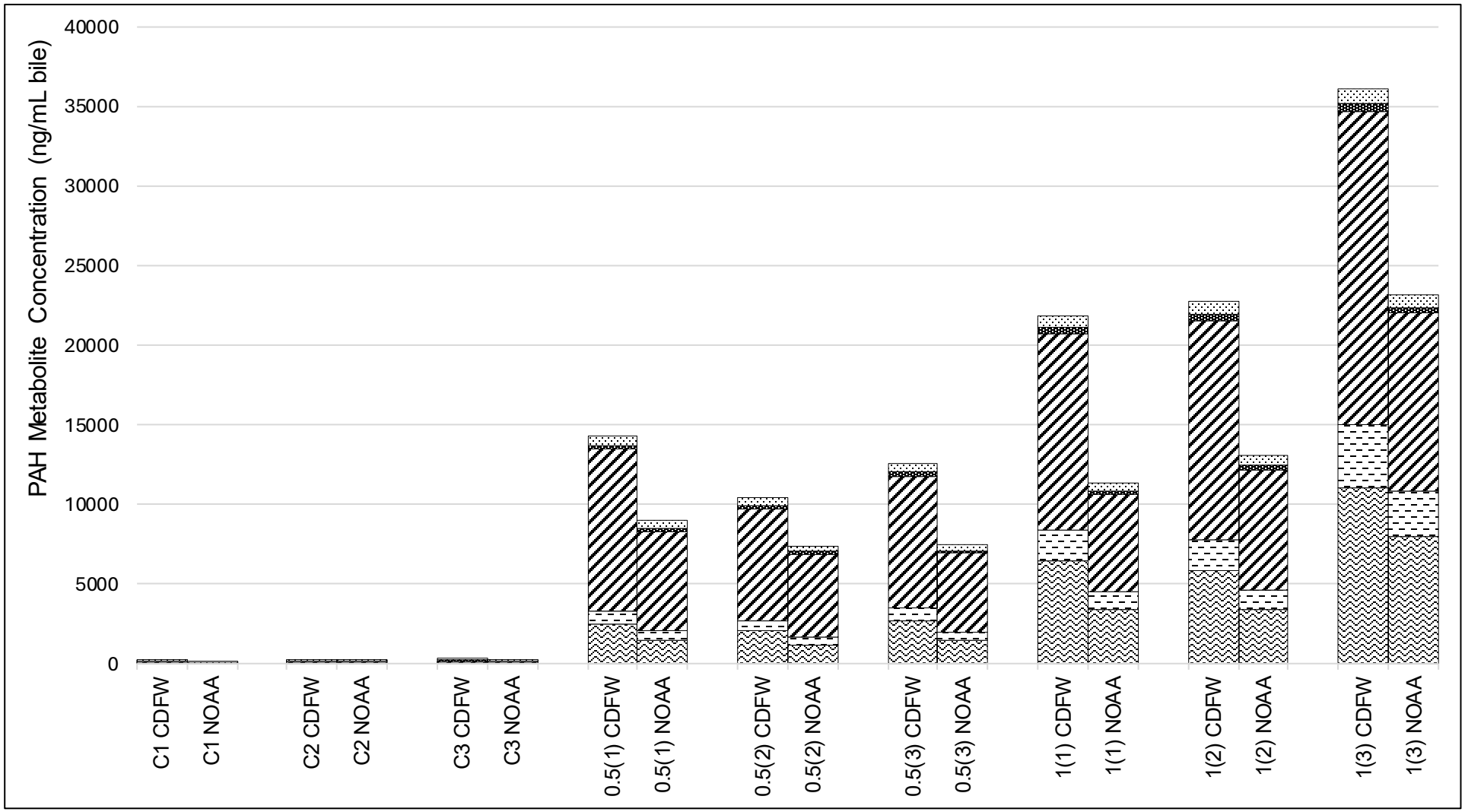

Figure 17. Test 3 replicate comparison of individual biliary PAH metabolite sample results for overlapping target analytes between the NOAA and CDFW laboratories.
C = control, 0.5 = 0.5 g/L loading, 1 = 1.0 g/L loading. 1-OH-N ( ), 2-OH-N ( ), 2-OH-F/ 3-OH-F ( ), 2-OH-P/ 3-OH-P ( ), 1-OH-P ( ).

Conclusions

The results demonstrate that topsmelt were not acutely affected by 1.0 g/L HEWAF of weathered oil, under static, aerated conditions. However, exposure to 1.0 g/L HEWAF of unweathered oil, with daily renewals and no aeration, did result in mortality and a reduced response to stimuli. Although exposure to 0.5 g/L and 1.0 g/L HEWAF of unweathered oil, with daily renewals and no aeration, did not result in mortality, a similar response to stimuli did occur for fish exposed to the higher HEWAF loading rate. The fish exposed to the two loading rates of unweathered oil were twice the size as those of other tests. This may have contributed to the non-reproduceable physical changes (snout redness) between tests with the same loading rate (1.0 g/L).

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