1 **Title**: Lionfish (*Pterois volitans*) as biomonitoring species for oil pollution effects in coral reef
2 ecosystems.
3
4

5 **Authors**: , Peter van den Hurk[1,6], Ian Edhlund[6], Ryan Davis[1], Jacob J. Hahn[2], Michel J.
6 McComb[1], Elizabeth L. Rogers[3], Emily Pisarski[4], Katy Chung[4], Marie DeLorenzo[5]
7
8 **Affiliation**:
9 [1] Department of Biological Sciences, College of Science, Clemson University
10 [2] Department of Genetics and Biochemistry, College of Science, Clemson University
11 [3] Department of Animal and Veterinary Sciences, College of Agriculture, Forestry and Life
12 Sciences, Clemson University
13 [4] CSS, Inc., under contract to NOAA
14 [5] NOAA, National Ocean Service, National Centers for Coastal Ocean Science, Charleston, SC
15 [6] Graduate Program in Environmental Toxicology, Clemson University
16

17 **Abstract:**
18

19 With oil spills, and other sources of aromatic hydrocarbons, being a continuous threat to coral
20 reef systems, and most reef fish species being protected or difficult to collect, the use of the
21 invasive lionfish (*Pterois volitans*) might be a good model species to monitor biomarkers in
22 potentially exposed fish in the Caribbean and western Atlantic. The rapid expansion of lionfish in
23 the Caribbean and western Atlantic, and the unregulated fishing for this species, would make
24 the lionfish a suitable candidate as biomonitoring species for oil pollution effects. However, to
25 date little has been published about the responses of lionfish to environmental pollutants. For
26 this study lionfish were collected in the Florida Keys a few weeks after Hurricane Irma, which
27 sank numerous boats resulting in leaks of oil and fuel, and during the winter and early spring
28 after that. Several biomarkers indicative of exposure to PAHs (bile fluorescence, cytochrome
29 P450-1A induction, glutathione S-transferase activity) were measured. To establish if these
30 biomarkers are inducible in PAH exposed lionfish, dosing experiments with different
31 concentrations of High Energy Water Accommodated Fraction of crude oil were performed. The
32 results revealed no significant effects in the biomarkers in the field collected fish, while the
33 exposure experiments demonstrated that lionfish did show strong effects in the measured
34 biomarkers, even at the lowest concentration tested (0.3% HEWAF, or 25 µg/l ΣPAH50). Based
35 on its widespread distribution, relative ease of collection, and significant biomarker responses in
36 the controlled dosing experiment, it is concluded that lionfish has good potential to be used as a
37 standardized biomonitoring species for oil pollution in its neotropical realm.
38
39

40 **Introduction:**
41 In late August through early September of 2017, Hurricane Irma swept through the Caribbean
42 with winds reaching up to 82.7 m/s. As the eye of the hurricane passed directly over the Florida
43 Keys, a large number of recreational and fishing boats that were moored in marinas and private
44 docks overturned and sank. Oil and fuel from these vessels leaked into the ocean, polluting

45 near shore environments, and potentially the reef communities further offshore. Reef
46 researchers and local recreational scuba divers were concerned these hydrocarbon leaks could
47 have an effect on the reefs in the Florida Keys.
48
49 Oil hydrocarbons can cause significant damage to reefs if the coral species are getting in
50 contact with the oil (Turner and Renegar, 2017). Oil can kill the coral, and can cause difficulties
51 in growth, reproduction, and development of the larvae. Not only coral species, but the entire
52 ecosystem of a reef, including fish, crustaceans and other invertebrates that live within the reef
53 ecosystem will suffer from oil spills. This can also cause health issues to any larger predatory
54 fish that consumes smaller exposed fish and can lead to trophic transfer up the food chain. The
55 Florida Keys reefs have been under enormous stress for several decades due to uncontrolled
56 wastewater discharges, introduced pathogens, recreational and commercial fishing pressure,
57 and increasing seawater temperature and acidification as a result of global climate change
58 (Futch et al., 2010; Ateweberhan et al., 2013; Kemp et al., 2018; Toth et al., 2019). Additional
59 stress by direct hurricane damage and indirect through the release of oil and fuel from sunken
60 boats may further deteriorate the reefs or hamper the recovery from other stressors.
61
62 To monitor the effects of environmental pollutants on aquatic and marine organisms, biomarkers
63 have been identified and implemented as early warning signals for the detrimental effects of
64 exposure to those pollutants (Van der Oost et al., 2003). In large animals, like sea mammals,
65 non-invasive techniques are available to measure biomarker responses without killing the
66 animal. However, in smaller organisms like most reef dwellers, tissue specimens need to be
67 collected after the animals are taken out of the water, which usually involves euthanizing the
68 organism. This creates a problem for researchers in highly protected reef environments; most
69 species are protected or have at least limited harvest time restrictions. To avoid these collection
70 restrictions, especially in rapid response projects that don't allow for lengthy permit applications,
71 the collection of undesired invasive species may be a solution to obtain biomarker results that
72 indicate exposure to, and effects of, released pollutants. In the Caribbean, the invasive lionfish
73 (*Pterois volitans*) may fulfill the requirements of an abundant coral reef species for which there
74 are no sampling restrictions.
75
76 The lionfish is an invasive species to the Caribbean, originally native to the Indo-Pacific region.
77 It was first noticed in 1985 off the Atlantic coast of Florida, and has gradually expanded into the
78 Caribbean and along the western Atlantic coasts (Bors et al., 2019). Although the species is a
79 voracious feeder on a large variety of smaller reef fish, recent reports question earlier
80 suggestions that this invasion would severely diminish reef fish diversity in the Caribbean
81 (Hackerott et al., 2017). The potential threat of the ecological effects of the expansion of lionfish
82 has spurred numerous round-up derbies by local scuba diver communities, with mixed results
83 (Usseglio et al., 2017).
84
85 The goal of this project was to evaluate if lionfish can be used as biomonitoring species for the
86 effects of oil spills in coral reef ecosystems. To achieve this goal, biomarkers that are typically
87 used to measure hydrocarbon exposure (bile fluorescence, ethoxyresorufin-O-deethylase
88 (EROD) activity, glutathione S-transferase (GST) activity) were measured in lionfish that were

89 collected at several time intervals after Hurricane Irma had created oil spills from sunken boats
90 in the Florida Keys. In addition, lionfish were exposed in controlled experiments to concentration
91 ranges of High-Energy Water Associated Fractions (HEWAFs) of crude oil to verify that these
92 biomarkers in this species are actually responsive to aromatic hydrocarbons. No published
93 papers were found on the effects of oil hydrocarbons on lionfish, which makes these studies
94 unique and relevant for monitoring the effects of future oil spills on coral reef species.
95
96
97 **Materials and Methods:**
98
99 *Fish and tissue collection*
100 Lionfish were collected by spearfishing from 11 locations in the Middle Keys area, south and
101 southeast of Marathon, Florida (FL) (Figure 1). The first collection trip was from October 14-16,
102 2017, and is labeled as "Fall", the second collection trip, labeled "Winter", was from December
103 14-16, 2017, and the third trip in "Spring" was from March 11-21, 2018. Exact sampling
104 locations, numbers of fish and size range are provided in Supplemental Information Table 1.
105 Locations were selected based on prior knowledge of lionfish availability, and to obtain a variety
106 of depth and distance to marinas as potential sources of oil pollution. Collected fish were kept
107 on ice until landing, and were measured for weight and body length before they were dissected
108 to remove their livers and gallbladders. Livers were wrapped in labeled aluminum foil, and
109 gallbladders were sealed in cryotubes. All samples were frozen in liquid nitrogen until arrival
110 back at the lab in Clemson, South Carolina (SC), where they were stored at -80□ until further
111 processing.
112
113 Fish for the first exposure experiment were collected from May 1-6, 2018; fish for the second
114 exposure experiment were collected March 25-29, 2019. Fish for the exposure experiments
115 were collected at several of the earlier mentioned locations south of Marathon, FL, with
116 handheld lobster nets, and were kept as mixed population in an outdoors flow through system in
117 Conch Key, Fl. Fish that were collected at deeper sites had their swimbladder vented with a
118 large needle to adjust them to sea surface atmospheric pressure. Fish were transported in an
119 aerated live well to the NOAA-NOS lab in Charleston SC, where they were acclimated to local
120 conditions for 3-7 days and fed a mixture of grass shrimp (*Palaemonetes pugio*) and
121 mummichogs (*Fundulus heteroclitus*) until transfer to the exposure tanks.
122

123

124
125

Figure 1. Sampling sites in the Florida Keys south of the island of Marathon; panel A shows
general area in south Florida, panel B shows detailed location of each individual site, numbered
1-11 from west to east. Geographical coordinates and depth of each location are provided in
Supplemental Information Table 1.

130

131

132

Experimental setup

For the 96h exposure experiments, four fish per treatment were individually housed in 10-liter
containers for the smaller fish (< 200 mm) and 35-liter tanks for the larger animals (>200 mm).
Seawater for all bioassays was acquired from Charleston Harbor estuary (N 32°45´11.52"; W
79°53´58.31"), filtered (5 µm), UV-sterilized, and activated carbon filtered. Tanks were covered
with aluminum foil, aerated using a 1 ml glass pipette attached to tubing, and fish were not fed
during the exposure. Fish were exposed to different concentrations of High Energy Water
Accommodated Fraction (HEWAF) of Louisiana Sweet Crude (LSC) oil. This 1 g/l HEWAF was
prepared by mixing 3.75 l of seawater (32 ppt) with fresh LSC oil (3.75 g by mass) in a
commercial blender, on low power, for 30 s. The mixture was transferred to a glass aspirator
bottle with the bottom outlet closed with Tygon tubing and a glass stopper. The mixture was
allowed to settle for 1 h in the dark, after which the bottom outlet was opened, and the HEWAF
was dispensed into a collection container, without disturbing the upper slick layer. The 100%
HEWAF was then diluted with seawater to achieve the exposure concentrations. The HEWAF
was diluted for the first experiment to exposure concentrations of 25%, 8.33% and 2.78%
HEWAF and a seawater control. For the second experiment the exposure concentrations were
lowered to 2.78%, 0.9% and 0.3% HEWAF and a seawater control. Treatments were renewed
after 48h. After the exposure, fish were euthanized in 1 g/l tricaine methanesulfonate (MS-222),
weighted and measured, dissected, and tissues were preserved as described above for the field
collected animals.

153

Chemical analysis

Water samples for the 100% HEWAF and all treatments (composite of four replicates) were
collected immediately after dosing (t =0 h and t =48h). Polynuclear aromatic hydrocarbon (PAH)
concentrations in the exposure tanks were analyzed according to standard NOAA-NOS

158 procedures. Briefly, samples were acidified to a pH of 2, isotopically labeled internal standards
159 were added, and extracted via liquid/liquid extraction with dichloromethane and hexane.
160 Extracts underwent a cleanup step using silica solid phase extraction (SPE) and spiked with a
161 recovery standard prior to analysis using gas chromatography mass spectrometry (GC/MS -
162 Agilent 6890/5973N). The GC/MS contained a DB17ms analytical column (60 m × 0.25 mm ×
163 0.25 µm) and was operated in selected ion monitoring mode. A total of 50 PAHs were analyzed,
164 including both parent and alkylated PAHs, and are reported as ΣPAH50.
165

166 *Bile analysis*
167 To measure PAHs that were absorbed and excreted by the fish, bile samples were analyzed for
168 fluorescence at excitation/emission wavelength pairs that are specific for 2-ring, and 5-ring
169 PAHs. Gall bladders of 4 fish per treatment were thawed, and bile was released into dark
170 microcentrifuge tubes. Bile volume was measured and recorded, and if less than 50 µl,
171 deionized water was added to bring the volume up to 50 µl. Several consecutive serial dilutions
172 (1:20, 1:33, 1:66 for the field samples, 1:12.5, 1:125, 1:250, 1:2500 for the experimental
173 samples) were prepared in dark microcentrifuge tubes using a 50:50 methanol:water solution.
174 Fluorescence of aromatic compounds (FACs) was then measured in three replicate aliquots
175 from each dilution at 290/335 nm, and 380/430 nm excitation/emission wavelengths on a BioTek
176 Synergy H1 plate reader (BioTek, Winooski, VT). Raw fluorescence data were plotted against
177 dilution, and the values of the highest dilution not showing inner filter effects were used for
178 further calculations. The FAC values were corrected using a methanol:water blank and
179 normalized to bile volume (van den Hurk, 2006).
180

181 *Liver enzyme activities*
182 Activities of two inducible enzymes that are involved in the metabolism of toxicants were
183 measured in liver homogenates. Livers were weighed and individually homogenized with a glass
184 Potter☐Elvehjem homogenizer in 2 ml of chilled homogenization buffer (van den Hurk, 2006).
185 Liver homogenates were then centrifuged at 10,000 x g and 4° C for 20 min, after which the
186 supernatant (S9 fraction) was divided into three aliquots for later determination of
187 ethoxyresorufin-O-deethylase (EROD) activity, glutathione S-transferase (GST) activity, and
188 total protein concentration. The EROD and GST aliquots were stored in a -80°C freezer and the
189 protein aliquot was stored in a -20°C freezer prior to analysis. Protein concentrations were
190 measured with a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL), using bovine
191 serum albumin (BSA) to prepare the standard curve.
192

193 For the EROD assay, liver S9 fractions were diluted to 1.0 mg/ml total protein concentration,
194 and 100 µl of diluted S9 fractions (in duplicate) were added to a black 96-well plate. The
195 reaction was started by adding 2.5 mM NADPH in 150 µl of reaction buffer (0.2% BSA, 5mM
196 MgCl2, 0.1 mM ethoxyresorufin) to the assay wells (Schreiber et al., 2006). The fluorescence
197 was then recorded at Ex 530, Em 585 nm in 5-10 min intervals over 30 min on a BioTek
198 Synergy H1 plate reader. A 1 mg/ml BSA sample was used in duplicate as a blank. A 7-step
199 dilution series of resorufin in methanol was used to generate a standard curve ranging from 0-
200 800 nM.
201

202 Activity of GST was measured as the conjugation of glutathione with 1-chloro-2,4-
203 dinitrobenzene (CDNB) by cytosolic protein (Mierzejewski et al., 2014). The total reaction
204 mixture of 250 µl contained 0.1 M HEPES buffer (pH 7.6), 1 mM glutathione (GSH), and 25 µg
205 of S9 protein. The reaction was started by adding CDNB (1 mM final concentration). Formation
206 of the CDNB conjugate was measured by taking absorption readings on a BioTek Synergy H1
207 plate reader at 20 s intervals for 2 min at 344 nm, and was quantified by using the molar
208 absorptivity of 9.6 mM-1 for the enzymatic product.
209
210 *Statistical analysis*
211
212 Biomarker data from field collected fish were analyzed for significant differences between sites
213 within seasons by two-way Analysis of Variance after log10 transformation. In addition, potential
214 seasonal differences were explored by pooling all log transformed data per season, and
215 analyzed with one-way Analysis of Variance. Data from the exposure experiments were also
216 log10 transformed for one-way Analysis of Variance, followed by Dunnett's post-hoc test to
217 compare treatments with the control. Differences were considered significant when $p<0.05$.
218 Data were analyzed with GraphPad Prism 4.03 (GraphPad, San Diego, CA).
219
220
221 **Results:**
222
223 *Field collected fish*
224
225 The bile fluorescence showed a fairly consistent pattern, with minor fluctuations between the
226 sampling locations and between seasons (Figures 2 and 3). Overall average was $69.2*10^3$ ($\pm$
227 37.1 S.D.) fluorescence units per µl bile for the 2-ring aromatic hydrocarbons, and $4.8*10^3$ (± 2.7
228 S.D.) units per µl for the 5-ring compounds. It appears that the Spring samples for the 2-ring
229 compounds may be slightly higher than the other seasons, but statistical analysis to identify any
230 significant differences between sites or seasons could not be performed because of lack of
231 replication; many sampling days had only one or a few fish per site, or none at all (Table 1
232 Supplemental Information). Even when all sites were pooled to look for seasonal differences, no
233 significant differences were detected.
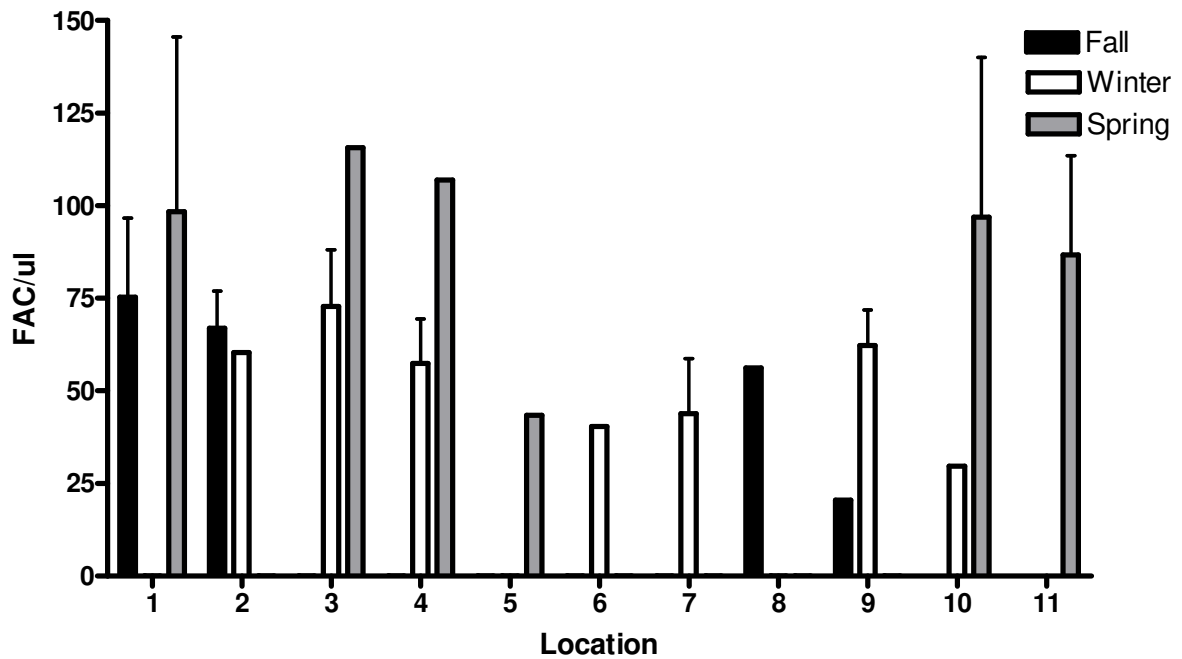
234
Figure 2. Fluorescence of 2-ring polynuclear aromatic hydrocarbons in bile samples from field
collected lionfish in Fall and Winter of 2017 and Spring of 2018. Fluorescent Aromatic
Compounds (FAC) in units*1000 per µl of bile. Sample locations arranged from west to east,
according to the map in Figure 1. Average values per site and season, with standard error.

239

240
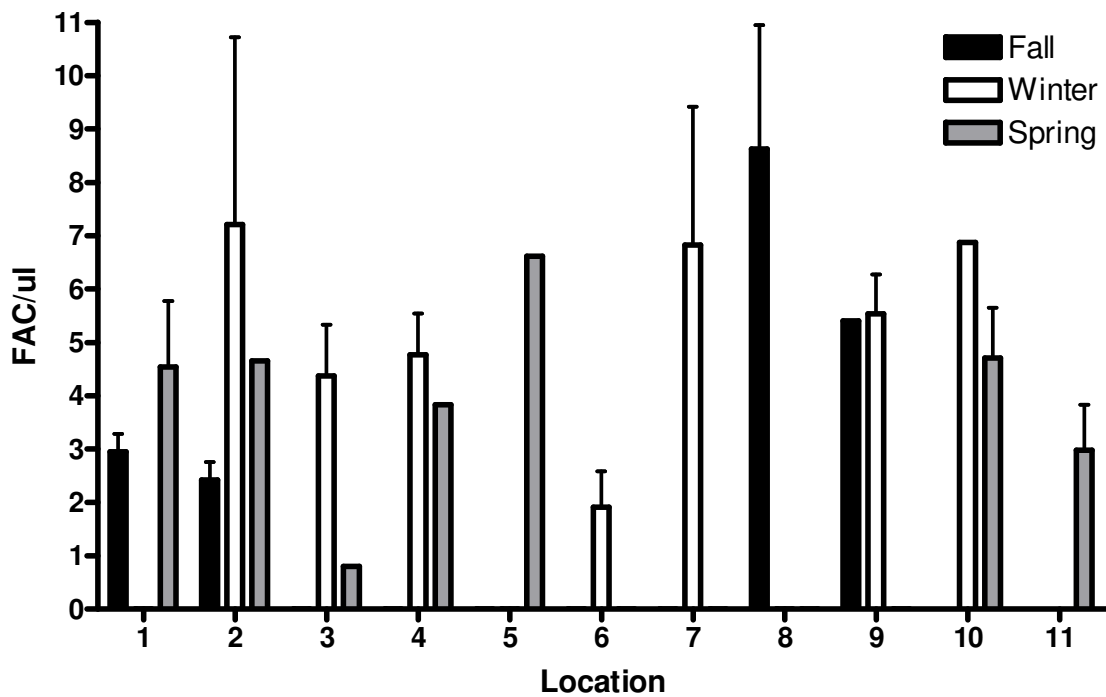
241 Figure 3. Fluorescence of 5-ring polynuclear aromatic hydrocarbons in bile samples from field
242 collected lionfish in Fall and Winter of 2017 and Spring of 2018. Fluorescent Aromatic
243 Compounds (FAC) in units*1000 per µl of bile. Sample locations arranged from west to east,
244 according to the map in Figure 1. Average values per site and season, with standard error.
245
246
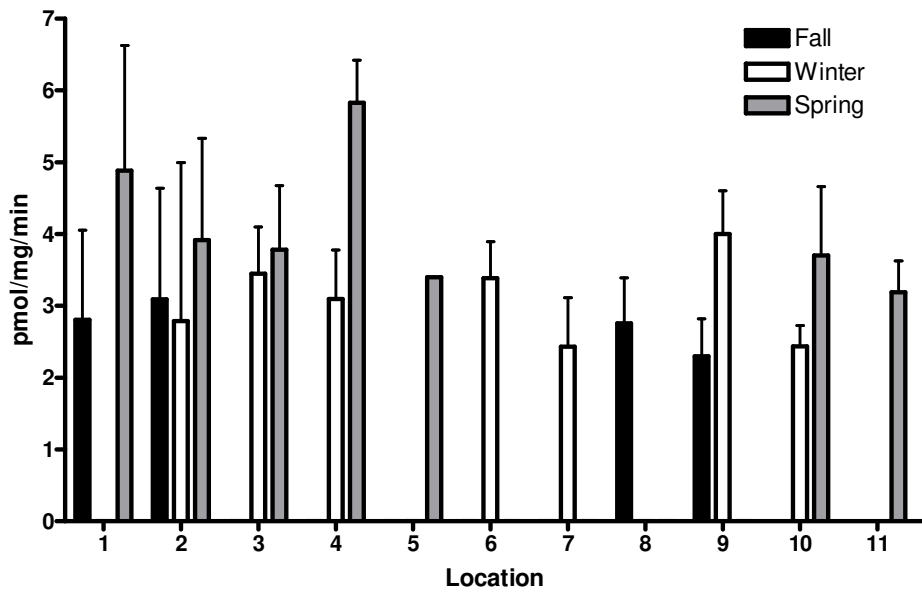
247
248 Figure 4. EROD activity in liver tissue from lionfish collected in the Florida Keys in Fall and
249 Winter of 2017 and Spring of 2018. Sample locations arranged from west to east, according to
250 the map in Figure 1. Average values per site and season, with standard error.
251
252
253 As for the bile fluorescence, EROD activity in lionfish livers showed a consistent pattern among
254 sites and seasons (Figure 4). Overall average was 3.42 pmol/mg/min (±1.75 S.D.), and even
255 though the Spring samples appear to be slightly increased compared to the other seasons, no
256 significant differences were detected between the seasons.
257
258

259
260 Figure 5. GST activity in liver tissue from lionfish collected in the Florida Keys in Fall and Winter
261 of 2017 and Spring of 2018. Sample locations arranged from west to east, according to the map
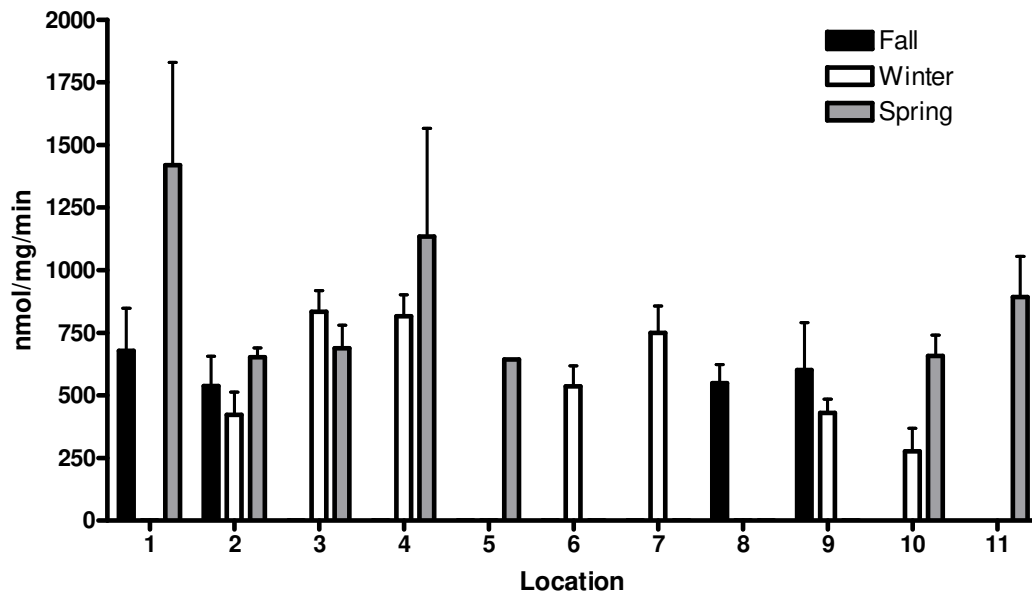262 in Figure 1. Average values per site and season, with standard error.
263
264 The GST activity in livers of the collected lionfish were fairly evenly distributed around the
265 overall average of 724 nmol/mg/min (± 369 S.D.) (Figure 5). As in the bile fluorescence data, it
266 appears that GST activity in some locations may be elevated, but because of limited sample
267 size for most locations, an analysis of variance could not be performed to confirm any significant
268 differences.
269
270 *Experimental results*
271
272 PAH concentrations:
273 The exposure concentrations for the first experiment were based on previous results obtained in
274 exposure experiments with the same HEWAF (DeLorenzo et al., 2018). In those studies, 25%
275 HEWAF was found to be approximately the LC_{50} for sheepshead minnow (*Cyprinodon*
276 *variegatus*). Not knowing what the expected toxicity of this HEWAF would be for lionfish, it was
277 decided to use 25% HEWAF as the highest concentration in the first experiment, with two lower
278 exposure concentrations according to a logarithmic sequence in the sublethal range (8.33 and
279 2.78 %). Based on the results of this first experiment, a second experiment was set up, for
280 which a lower dose range was selected, with the highest nominal concentration the same as the
281 lowest in the first experiment (2.78%), followed by log scale dilutions of 0.9 and 0.3 % HEWAF.
282 This resulted in a ΣPAH50 range of 24.7 – 2727.6 µg/l over both experiments (Table 1).
283 Concentrations of individual PAHs and alkylated PAHs in the HEWAF and the tested dilutions
284 are included in the Supplemental Information Tables 2 and 3. Observations from the first
285 experiment suggest that the highest concentration used was close to the LC_{50} for lionfish,

286 because two animals in this treatment group died before the end of the 96h exposure time.
287 Therefore n=2 for the highest concentration in the first experiment.
288
289

% HEWAF	Experiment 1	Experiment 2
100	6177.7	8563.8
25	2727.6	
8.33	513.3	
2.78	179.0	222.1
0.9		90.5
0.3		24.7

290
291 Table 1. Total PAH concentrations in HEWAF dilutions used for both exposure experiments.
292 ΣPAH50 concentrations in µg/l, mean of HEWAFs prepared at t=0h and t=48h for each
293 experiment.
294
295
296
297
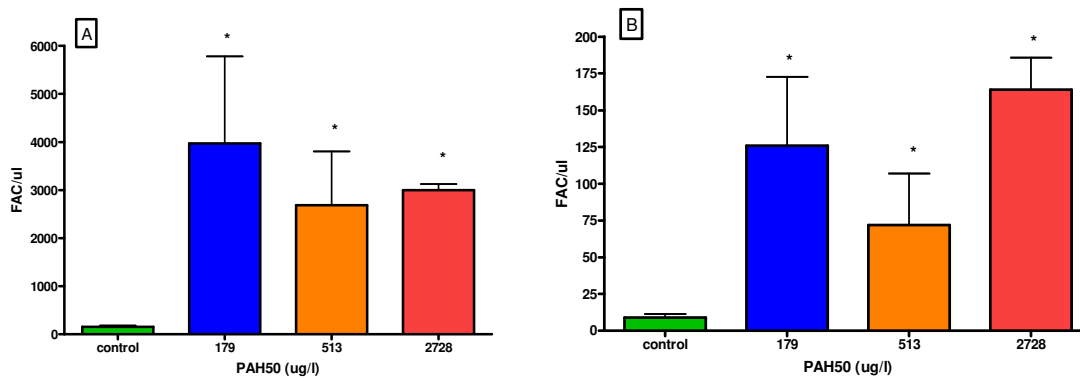
298
299
300 Fig 6. Fluorescence of 2-ring (Panel A) and 5-ring (Panel B) polynuclear aromatic hydrocarbons
301 in bile samples from HEWAF exposed lionfish in experiment 1. Fluorescent Aromatic
302 Compounds (FAC) in units*1000 per µl of bile for each treatment group, indicated by actual
303 ΣPAH50 concentration in exposure system. Average values, with standard error.
304
305
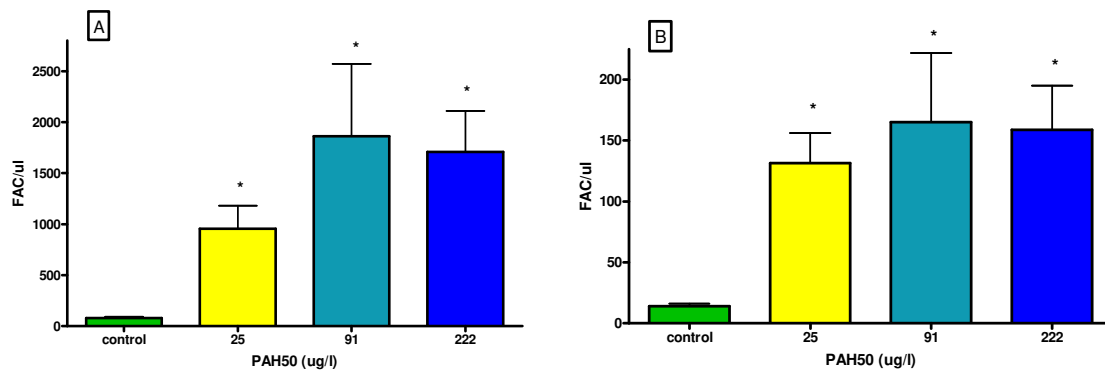
306
307 Fig 7. Fluorescence of 2-ring (Panel A) and 5-ring (Panel B) polynuclear aromatic hydrocarbons
308 in bile samples from HEWAF exposed lionfish in experiment 2. Fluorescent Aromatic
309 Compounds (FAC) in units*1000 per µl of bile for each treatment group, indicated by actual
310 ΣPAH50 concentration in exposure system. Average values (n=4, except n=2 for highest
311 concentration in experiment 1), with standard error.
312
313 After absorption, and potentially metabolism, of PAHs, these fluorescent compounds can be
314 excreted into the bile. Measuring bile fluorescence provides a measure of exposure to PAHs.
315 The fluorescence of multi-ring structures like PAHs is dependent on specific excitation and
316 emission wavelengths. Reported here are the fluorescence units that are specific for 2-ring and
317 5-ring PAHs. The data from the first experiment showed that for both the 2-ring and the 5-ring
318 PAHs there were highly significant differences between the treated fish and the control fish (p <
319 0.001 and p < 0.01 respectively), but no significant differences between the HEWAF exposed
320 treatment groups (Figure 6). This indicates that at the lowest concentration, maximum levels of
321 PAHs were present in the bile. In the second experiment, the pattern of results was the same in
322 that all treatment groups were significantly higher than the control (p < 0.001), but there were no
323 significant differences between the treatment groups (Figure 7). Although the fluorescence in
324 the lowest exposure concentration (26 µg/l) was lower than in the higher treatment groups for
325 both the 2-ring and 5-ring PAHs, these differences were not significant.
326
327
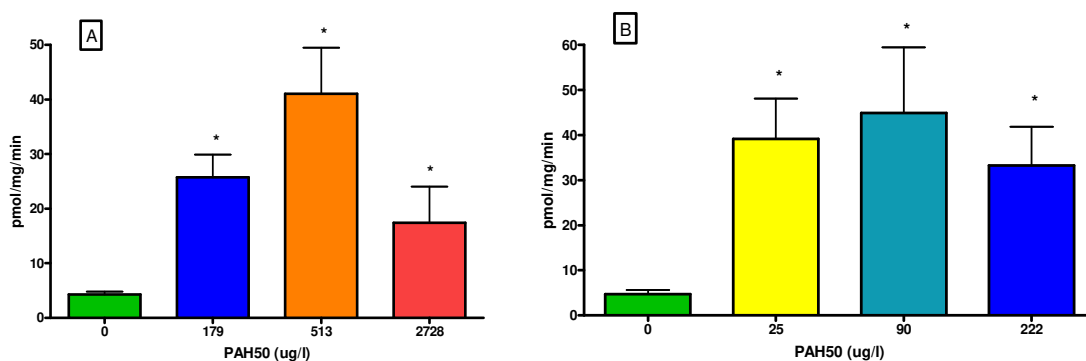
328
329 Figure 8. EROD activity in liver homogenates from HEWAF exposed lionfish. Results from
330 Experiment 1 (Panel A) and Experiment 2 (Panel B). EROD activity in pmol/mg/min for each

treatment group, indicated by actual ΣPAH50 concentration in exposure system. Average values, with standard error. Asterisks indicate significant differences from the control (p<0.05).

The measured EROD activity in experiment 1 was significantly increased in all treatment groups compared to the control, but there were no significant differences between the exposed treatment groups (Figure 8). The lower activity in the highest treatment (2728 µg/l) may be a result of the morbidity in these animals, as two animals in this treatment died prematurely. Therefore, n=2 in this treatment. In the second experiment, with lower exposure concentrations, again all treatment groups had significantly higher EROD activity than the control, up to 10 times higher, but no significant differences were observed between the treatment groups. It is noteworthy that even at the lowest exposure concentration of 25 µg/l ΣPAH50 there was a maximum induction of EROD activity.
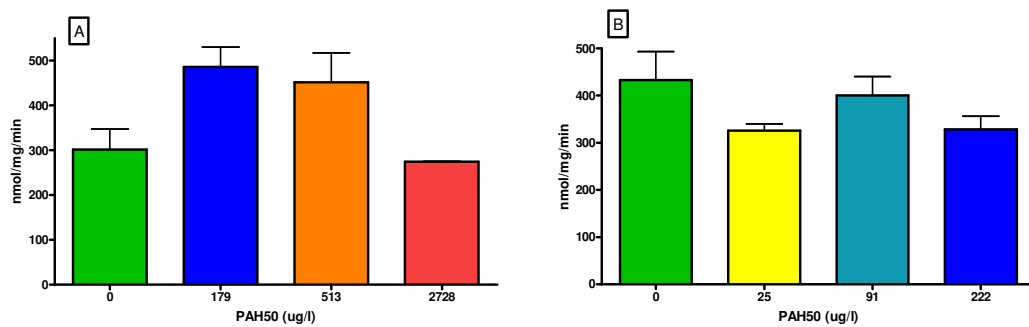
Figure 9. GST activity in liver homogenates from HEWAF exposed lionfish. Results from Experiment 1 (Panel A) and Experiment 2 (Panel B). GST activity in nmol/mg/min for each treatment group, indicated by actual ΣPAH50 concentration in exposure system. Average values with standard error.

The measured GST activity in the HEWAF exposed fish was not significantly different from the control in either one of the experiments (Figure 9). The lower activity in the highest treatment group of experiment 1 may be a result of morbidity in these fish, as was seen in the EROD activity in these fish.

Discussion and conclusions:

The goals of this study were to investigate if lionfish could be used as biomonitoring species for oil spills in coral reef ecosystems, and specifically if oil and diesel fuel that leaked from boats that were sunk when hurricane Irma hit the Florida Keys in August 2017 had an effect on lionfish in that area. Fish were captured in an area south of Marathon Key within a few weeks after the hurricane came through, but the results did not show any overall differences for the biomarkers measured when compared with fish that were collected several months later in December 2017 or March 2018.

367   While there could be a variety of reasons that no significant differences were found between the
368   seasons or between the sites, it was unknown if the biomarkers that were measured are actually
369   responsive in lionfish when they are exposed to mineral oil compounds. We therefore set up
370   experiments in which lionfish were exposed to a seawater extract of Louisiana crude oil. The
371   results of these experiments showed that lionfish do absorb the oil related PAHs and excrete
372   them into the bile, as measured through the bile fluorescence biomarker. This biomarker has
373   found widespread application, and is considered a reliable indicator of exposure to PAHs (Beyer
374   et al., 2010; Kamann et al., 2017).

376   As a result of the experimental oil exposure, an increase in cytochrome P450-1A was observed,
377   as measured through the EROD assay, which is an indicator for the cytochrome P450 isoform
378   that is specific for aromatic hydrocarbons. Even at the lowest ΣPAH50 concentration of 25 µg/l a
379   10 times induction over the control was measured, which has been observed for other species
380   as well (Whyte et al., 2000; Schlenk et al., 2008). Follow up studies could be performed at even
381   lower concentrations to obtain a classical dose-response curve. However, for the proof of
382   concept purpose of this study the near maximum EROD response observed at 25 µg/l, which
383   corresponds to 3 mg/l of crude oil, indicates that increased EROD activity in lionfish should be
384   expected in field situations where fish were recently exposed to oil spills. Environmental
385   concentrations of PAHs around oil spills are highly variable because of rapid dispersion and
386   differential fate of different groups of PAHs, but concentrations of up to 65.8 µg/l of total
387   hydrocarbons were measured in plumes associated with the *Deepwater Horizon* oil spill (Spier
388   et al., 2013).

390   The GST activity in lionfish was not significantly changed as a result of exposure to the HEWAF.
391   The assay used to measure GST activity is not specific for individual GST isoforms, but more a
392   general indicator for overall GST activity. Nevertheless, some have found that overall GST
393   activity can be induced as a result of oil exposure (Kerambrun et al., 2012; Nahrgang et al.,
394   2010); but others have argued that the GST biomarker is very species specific (Smeltz et al.,
395   2017; van den Hurk et al., 2017). Because the overall GST activity, as measured with the CDNB
396   assay, is not specific for individual GST isoforms, effects on a lesser expressed isoforms may
397   be masked by more abundant, non-responsive isoforms in selected species.

399   Comparing the results of the field collected fish with data from the exposure experiments
400   demonstrated that all the measured biomarkers in the field collected fish fall within the normal
401   background range. The exposure experiments showed that when lionfish are exposed to oil
402   compounds, a significant increase in bile fluorescence and EROD activity can be expected.
403   Because these increases were not observed in the field collected fish, it is concluded that they
404   were not exposed to oil compounds in the period before the first sampling period.

406   The opportunity to sample soon after an oil spill or other environmental disaster may be
407   hampered by logistic constraints. The passage of hurricane Irma through the Florida Keys
408   created a major disruption of infrastructure and loss of property (https://www.monroecounty-
409   fl.gov/726/Hurricane-Irma-Recovery), and thanks to generous support by local concerned
410   residents, we were able to collect lionfish at several locations south of Marathon, FL, only four

411 weeks after the hurricane had passed through the area. The fact that we didn't observe any
412 toxicological effects in the lionfish that were collected at that point could be attributed to the time
413 period that had passed between the disaster, and its associated oil and fuel spills, and the
414 measurements of biomarkers in fish. However, several studies, reviewed in Lee and Anderson
415 (2005), showed that cytochrome P450-1A activities may be elevated up to four months, or
416 longer, in fish exposed to oil compounds. This would indicate that if the lionfish in our research
417 area were exposed to significant amounts of oil, an increased EROD activity was to be
418 expected. However, depending on the composition of the oil, as was demonstrated after the
419 *Deepwater Horizon* spill, the environmental fate of oil compounds in the marine environment
420 can be quite unpredictable. Smaller, more volatile compounds evaporate or are metabolized by
421 microorganisms, while the heavier compounds form tar balls and end up in coastal sediments
422 (Mulabagal et al., 2013). Based on our results for both the bile fluorescence and EROD
423 induction it can be assumed that the reef systems south of Marathon, FL, were not impacted by
424 oil and fuel leaks originating from sunken ships in the surrounding marinas, and that the lack of
425 a response is not due to a potential four week recovery period between the occurrence of the
426 hurricane and the first sampling period.
427
428 The exposure experiments clearly demonstrated that lionfish are sensitive to oil pollution, and
429 biomarkers like bile fluorescence and EROD activity are responsive to oil exposure in this
430 species. Very few studies have been published on the ecotoxicology of lionfish, including
431 studies on the accumulation of mercury, and the effects of the pesticide chlordecone (Huge et
432 al., 2014; Ritger et al., 2018; Charlotte et al., 2016). Some locations in the study by Ritger et al.
433 (2018) were close to a large petroleum refinery on the island of Curaçao, which could be a
434 source of PAHs. But no information on potential oil or PAH effects were reported, and further
435 inquiry with the authors did not reveal any additional observations in lionfish of effects that could
436 be attributed to exposure to oil compounds. In a recent study by Horricks et al. (2019) results
437 are reported that indicate that lionfish are bioaccumulating residues of ultraviolet filters that are
438 commonly used in sunscreen lotions, and that lionfish may therefore be a useful sentinel
439 species for monitoring these organic ultraviolet filters in the Caribbean Sea.
440
441 The 2010 *Deepwater Horizon* oil spill released millions of gallons of crude oil into the Gulf of
442 Mexico (Beyer et al., 2016). To assess the ecotoxicological effects of this spill, a large number
443 of studies were undertaken to measure the effects of spilled oil on different life forms in the Gulf
444 of Mexico. In a recent publication, a summary of the 50+ fish studies published so far were
445 reviewed (Pasparakis et al., 2019), but only one study reported the effects on reef fish
446 (Johansen et al, 2017). In this study, larvae of *Chromis*, *Pomacentrus*, and *Lethrinus* were
447 exposed to oil and monitored for behavioral impairments, but no information on potential effects
448 in lionfish or related species were reported.
449
450 Based on the results from this study it can be concluded that lionfish is a suitable species for
451 monitoring the ecotoxicological effects of oil pollution incidents. Future research into the
452 responses of this species to other mixtures of PAHs than HEWAFs, as found for instance in
453 diesel fuel, should further support the preliminary results obtained in this study. A benefit of
454 using this species in future impact assessments is the widespread occurrence in the Caribbean,

Gulf of Mexico and western Atlantic coastal zone. Because this is an invasive species there are no restrictions on capturing lionfish, and their relatively slow swimming speed, easy recognition, and tendency to linger among coral reef structures makes them easy to collect (Chaves et al., 2016). This opens up the possibility for involving SCUBA divers as citizen scientists and have them assist in collecting sufficient numbers of animals per location. Numerous lionfish derby events are organized on a regular basis throughout the newly established range in the Western hemisphere; which would expand possibilities for citizen scientists to be involved in monitoring oil spill events beyond the already established network of commercial fishermen and fishing communities (Sullivan et al., 2018).

References:

Ateweberhan M, Feary DA, Keshavmurthy S, Chen A, Schleyer MH, Sheppard CR. (2013) Climate change impacts on coral reefs: synergies with local effects, possibilities for acclimation, and management implications. Mar Pollut Bull 74(2):526-39

Beyer J, Jonsson G, Porte C, Krahn MM, Ariese F. (2010) Analytical methods for determining metabolites of polycyclic aromatic hydrocarbon (PAH) pollutants in fish bile: A review. Environ Toxicol Pharmacol. 2010 Nov;30(3):224-44

Beyer J, Trannum HC, Bakke T, Hodson PV, Collier TK. (2016) Environmental effects of the Deepwater Horizon oil spill: A review. Mar Pollut Bull 110(1):28-51

Bors EK, Herrera S, Morris JA, Shank TM. (2019) Population genomics of rapidly invading lionfish in the Caribbean reveals signals of range expansion in the absence of spatial population structure. Ecol Evol. 9(6):3306-3320

497 Charlotte DR, Yolande BN, Cordonnier S, Claude B. (2016) The invasive lionfish, Pterois
498 volitans, used as a sentinel species to assess the organochlorine pollution by chlordecone in
499 Guadeloupe (Lesser Antilles). Mar Pollut Bull. 107(1):102-106

501 Chaves LC, Hall J, Feitosa JL, Côté IM. (2016) Photo-identification as a simple tool for studying
502 invasive lionfish Pterois volitans populations. J Fish Biol 88(2):800-4

504 DeLorenzo ME, Key PB, Chung KW, Pisarski E, Shaddrix B, Wirth EF, Pennington PL, Wade J,
505 Franco M, Fulton MH. (2018) Comparative Toxicity of Two Chemical Dispersants and Dispersed
506 Oil in Estuarine Organisms. Arch Environ Contam Toxicol. 2018 Apr;74(3):414-430

508 Futch JC, Griffin DW, Lipp EK. (2010) Human enteric viruses in groundwater indicate offshore
509 transport of human sewage to coral reefs of the Upper Florida Keys. Environ Microbiol.
510 12(4):964-74

512 Hackerott S, Valdivia A, Cox CE, Silbiger NJ, Bruno JF. (2017) Invasive lionfish had no
513 measurable effect on prey fish community structure across the Belizean Barrier Reef. PeerJ.
514 5:e3270

516 Horricks RA, Tabin SK, Edwards JJ, Lumsden JS, Marancik DP. (2019) Organic ultraviolet
517 filters in nearshore waters and in the invasive lionfish (Pterois volitans) in Grenada, West Indies.
518 PLoS One. 14(7):e0220280

520 Huge DH, Schofield PJ, Jacoby CA, Frazer TK. (2014) Total mercury concentrations in lionfish
521 (*Pterois volitans/miles*) from the Florida Keys National Marine Sanctuary, USA. Mar Pollut Bull.
522 78(1-2):51-5

524 Johansen JL, Allan BJM, Rummer JL, Esbaugh AJ. (2017) Oil exposure disrupts early life-
525 history stages of coral reef fishes via behavioural impairments.Nat Ecol Evol. 2017
526 Aug;1(8):1146-1152. doi: 10.1038/s41559-017-0232-5. Epub 2017 Jul 17. Erratum in: Nat Ecol
527 Evol. 2017 Sep;1(9):1412.

529 Kammann U, Akcha F, Budzinski H, Burgeot T, Gubbins MJ, Lang T, Le Menach K, Vethaak
530 AD, Hylland K. (2017) PAH metabolites in fish bile: From the Seine estuary to Iceland. Mar
531 Environ Res.124:41-45

533 Kemp KM, Westrich JR, Alabady MS, Edwards ML, Lipp EK. (2018) Abundance and Multilocus
534 Sequence Analysis of Vibrio Bacteria Associated with Diseased Elkhorn Coral (Acropora
535 palmata) of the Florida Keys. Appl Environ Microbiol. 84(2). pii: e01035-17

537 Kerambrun E, Le Floch S, Sanchez W, Thomas Guyon H, Meziane T, Henry F, Amara R.
538 (2012) Responses of juvenile sea bass, Dicentrarchus labrax, exposed to acute concentrations
539 of crude oil, as assessed by molecular and physiological biomarkers. Chemosphere 87(7):692-
540 702

541

542 Lee RF, Anderson JW. (2005) Significance of cytochrome P450 system responses and levels of
543 bile fluorescent aromatic compounds in marine wildlife following oil spills. Mar Pollut Bull.
544 50(7):705-23

545

546 Mierzejewski J, Haney DC, van den Hurk P. (2014) Biomarker responses in sunfish species and
547 largemouth bass from the Saluda River, South Carolina. Ecotoxicol Environ Saf. 2014
548 Dec;110:8-15

549

550 Mulabagal V, Yin F, John GF, Hayworth JS, Clement TP. (2013) Chemical fingerprinting of
551 petroleum biomarkers in Deepwater Horizon oil spill samples collected from Alabama shoreline.
552 Mar Pollut Bull 70(1-2):147-54

553

554 Nahrgang J, Camus L, Gonzalez P, Jönsson M, Christiansen JS, Hop H. (2010) Biomarker
555 responses in polar cod (Boreogadus saida) exposed to dietary crude oil. Aquat Toxicol. 2010
556 Jan 21;96(1):77-83

557

558 Pasparakis C, Esbaugh AJ, Burggren W, Grosell M. (2019) Impacts of deepwater horizon oil on
559 fish.Comp Biochem Physiol C Toxicol Pharmacol. 224:108558. doi: 10.1016/j.cbpc.2019.06.002

560

561 Ritger AL, Curtis AN, Chen CY. (2018) Bioaccumulation of mercury and other metal
562 contaminants in invasive lionfish (*Pterois volitans/miles*) from Curaçao. Mar Pollut Bull. 131(Pt
563 A):38-44

564

565 Schlenk D, Celander M, Gallagher E, George S, James M, Kullman S, Van den Hurk P, Willett
566 K. (2008). Biotransformation in Fishes. In: Toxicology of fishes. D. Hinton, R. DiGiulio (eds).
567 CRC Press, Boca Raton, Fl. pp 153-234

568

569 Schreiber EA, Otter RR, Van den Hurk P. (2006) A biomarker approach to measure biological
570 effects of contaminant exposure in largemouth bass from Lake Conestee, South Carolina,
571 U.S.A. Environ Toxicol Chem 25(7):1926-32

572

573 Smeltz M, Rowland-Faux L, Ghiran C, Patterson WF 3rd, Garner SB, Beers A, Mièvre Q, Kane
574 AS, James MO. (2017) A multi-year study of hepatic biomarkers in coastal fishes from the Gulf
575 of Mexico after the Deepwater Horizon Oil Spill. Mar Environ Res 129:57-67

576

577 Spier C, Stringfellow WT, Hazen TC, Conrad M. (2013) Distribution of hydrocarbons released
578 during the 2010 MC252 oil spill in deep offshore waters. Environ Pollut. 173:224-30

579

580 Sullivan J, Croisant S, Howarth M, Rowe GT, Fernando H, Phillips-Savoy A, Jackson D,
581 Prochaska J, Ansari GAS, Penning TM, Elferink C; Community Partner Authors: Louisiana
582 Environmental Action Network, United Houma Nation, Bayou Interfaith Shared Community
583 Organizing, Dustin Nguyen-Vietnamese Community Partner, Center for Environmental &
584 Economic Justice, and Alabama Fisheries CooperativeProject Community Scientist Author:

585 Wilma Subra. (2018) Building and Maintaining a Citizen Science Network With Fishermen and
586 Fishing Communities Post Deepwater Horizon Oil Disaster Using a CBPR Approach. New
587 Solut. 28(3):416-447
588
589 Toth LT, Stathakopoulos A, Kuffner IB, Ruzicka RR, Collela MA, Shinn EA. (2019) The
590 unprecedented loss of Florida's reef-building corals and the emergence of a novel coral-reef
591 assemblage. Ecology e02781. doi: 10.1002/ecy.2781
592
593 Turner NR, Renegar DA. (2017) Petroleum hydrocarbon toxicity to corals: A review. Mar Pollut
594 Bull. 119(2):1-16
595
596 Usseglio P, Selwyn JD, Downey-Wall AM, Hogan JD. (2017) Effectiveness of removals of the
597 invasive lionfish: how many dives are needed to deplete a reef? PeerJ. 5:e3043
598
599 van den Hurk P, Gerzel LE, Calomiris P, Haney DC. (2017) Phylogenetic signals in
600 detoxification pathways in Cyprinid and Centrarchid species in relation to sensitivity to
601 environmental pollutants. Aquat Toxicol. 188:20-25
602
603 van den Hurk P. (2006) Bile fluorescence, heme oxygenase induction, and increased biliverdin
604 excretion by mixtures of environmental toxicants. Aquat Toxicol. 77(2):202-9
605
606 van der Oost R, Beyer J, Vermeulen NP. (2003) Fish bioaccumulation and biomarkers in
607 environmental risk assessment: a review. Environ Toxicol Pharmacol. 2003 Feb;13(2):57-149
608
609 Whyte JJ, Jung RE, Schmitt CJ, Tillitt DE. (2000) Ethoxyresorufin-O-deethylase (EROD) activity
610 in fish as a biomarker of chemical exposure. Crit Rev Toxicol. 2000 Jul;30(4):347-570. Review
611
612
613
614
615 **Supplemental information:**
616
617 Supplemental Table 1. Sampling locations with depth and geographical coordinates, number of
618 fish collected per season, and size range of collected fish (mm).
619
620

Site	Depth	Latitude	Longitude	Fall (n)	Winter (n)	Spring (n)	Size Range (mm)
50 live	50	24.709733	-80.856867	3	6	0	195 - 252
Cannon	20	24.723100	-80.861733	2	0	0	187 - 206
Delta Shoal	20	24.631983	-81.090567	2	0	3	125 – 300

Grouper Trail	60	24.684783	-80.921867	0	7	7	115 - 240
Kissing Grunts	30	24.687217	-80.924150	0	4	4	115 - 250
Long Key Ledge	30	24.717930	-80.844680	0	1	5	110 - 220
Lost n Found	30	24.709367	-80.870100	0	7	0	87 - 255
Porkfish	30	24.700500	-80.893150	0	4	0	205 – 250
Thunderbolt	115	24.661200	-80.963283	2	2	2	131 - 276
Deep Drop	65	24.732090	-80.790810	0	0	8	120 – 280
West Turtle Shoals	18	24.720450	-80.927500	0	0	5	110-245

621
622
623
624 Based on Wilcox et al. (2018) we assumed that all fish belonged to the same species
625 continuum, and did not attempt to make distinctions between *P. volitans* and *P. miles*.
626 Population structure analysis showed that one year old lionfish are around 150 mm, and
627 because this species rarely gets older than 3 year old, all larger individuals are mostly 2 year old
628 (Johnson and Swenarton, 2016). Gender could not be established for the fish because they
629 were collected outside the spawning season, and therefore the gonads were inconspicuous.
630 Secondary sex characteristics that could have facilitated gender determination were not
631 observed in the collected fish.
632
633
634 Johnson EG, Swenarton MK. Age, growth and population structure of invasive lionfish (Pterois
635 volitans/miles) in northeast Florida using a length-based, age-structured population model.
636 PeerJ. 2016 Dec 1;4:e2730
637
638 Wilcox CL, Motomura H, Matsunuma M, Bowen BW. Phylogeography of Lionfishes (Pterois)
639 Indicate Taxonomic Over Splitting and Hybrid Origin of the Invasive Pterois volitans. J Hered.
640 2018 Feb 14;109(2):162-175
641
642
643 Supplemental Table 2. PAH concentrations in 100% HEWAF, and tested dilutions in Experiment
644 1, at beginning of exposure (t=0) and after 48 hours (t=48). All units in µg/L.
645

Chemical	100x t0	100x 48h	25x t0	8.3x t0	8.3x 48h	2.7x t0	2.7x 48h	0x t0
1,2-Dimethylnaphthalene	65.475	35.981	14.5305	1.246	2.98333	1.5663	0.9528	0.01801
1,3+1,6-Dimethylnaphthalene	644.915	373.978	147.545	13.8028	31.1883	16.3731	10.7899	0.08339
1,4,6- and 2,3,6-Trimethylnaphthalene	258.916	163.874	197.47	21.3968	14.8493	7.4395	4.449	0.08521
1,4-Dimethylnaphthalene	82.913	49.31	21.4978	1.53083	3.70033	2.1458	1.5069	0.04438

1,5-Dimethylnaphthalene	85.779	49.834	19.9225	1.70183	3.99283	2.1622	1.412	0.01377
1,6,7 Trimethylnaphthalene	217.425	148.59	198.55	18.3923	11.63	6.1285	4.007	0.05042
1,8-Dimethylnaphthalene	47.69	25.018	11.527	1.28467	0.58263	0.2503	0.2503	0.2503
11H-Benzo(a)fluorene	3.359	2.076	1.0425	0.29567	0.17683	0.1144	0.0635	0.01497
11H-Benzo(b)fluorene	11.831	9.043	3.34175	1.19983	0.74867	0.4559	0.2636	0.01993
1-Methylanthracene	4.92	3.729	1.38025	0.478	0.27283	0.1311	0.1248	0.00928
1-Methyldibenzothiophene	9.541	5.82	2.724	0.92183	0.84	0.0889	0.1826	0.00655
1-Methylfluorene	113.071	81.128	30.6433	10.3428	13.7543	3.4739	2.2059	0.01044
1-Methylnaphthalene	273.385	264.532	83.5343	26.9375	16.6403	12.3353	7.7184	0.14038
1-Methylphenanthrene	73.358	48.037	19.7655	6.61317	4.41417	2.2085	1.5212	0.01175
2,6+2,7-Dimethylnaphthalene	179.944	155.945	63.2415	17.446	15.4138	6.9251	3.6658	0.05242
2-Methylanthracene	6.648	0.02896	2.05075	0.76783	0.0051	0.00942	0.00285	0.01306
2-Methyldibenzothiophene	7.202	4.545	2.114	0.72533	0.61717	0.0684	0.1594	0.01434
2-Methylnaphthalene	489.629	365.143	126.863	40.5025	27.5885	17.115	10.048	0.2169
2-Methylphenanthrene	107.011	74.223	28.3285	9.69933	5.00733	3.4682	2.3477	0.02033
3-Methylcholanthrene	0.69763	0.20246	0.16411	0.11283	0.08074	0.04156	0.01519	0.05576
3-Methylphenanthrene	103.138	67.87	27.528	9.1185	5.65367	3.0054	2.0014	0.00757
4H-Cyclopenta[def]phenathrene	0.0285	0.02803	0.02004	0.01896	0.00496	0.00901	0.00274	0.00929
4-Methyldibenzothiophene	28.646	17.942	8.12675	2.77533	1.918	0.2604	0.5371	0.00677
9,10-Dimethylanthracene	0.07276	0.07158	0.05116	0.04841	0.01266	0.023	0.00699	0.02371
9-Methylanthracene	0.03194	0.03209	0.02271	0.02164	0.00565	0.01044	0.00316	0.01447
9-Methylphenanthrene	101.471	64.968	27.0108	8.88167	5.90383	2.9352	1.9607	0.01141
Acenaphthene	0.24769	0.25854	0.05593	0.16466	0.13279	0.02077	0.02148	0.02736
Acenaphthylene	15.3	9.374	4.0775	1.60267	0.92783	0.4105	0.2778	0.00521
Anthanthrene	0.55009	0.17856	0.13743	0.0717	0.0601	0.03128	0.0132	0.03752
Anthracene	0.02156	0.02121	0.01516	0.01434	0.00375	0.00682	0.00207	0.00703
Benzo(a)anthracene	2.901	1.658	0.82375	0.27917	0.15317	0.0871	0.052	0.00635
Benzo(a)pyrene	0.576	0.315	0.16075	0.02403	0.04712	0.02369	0.016	0.02806
Benzo(b)fluoranthene	1.5	1.198	0.421	0.2125	0.11017	0.0469	0.0441	0.01649
Benzo(e)pyrene	2.665	1.75	0.72575	0.21483	0.16667	0.0785	0.0544	0.01328
Benzo(g,h,i)perylene	0.435	0.298	0.13625	0.03633	0.01812	0.00943	0.005	0.01131
Benzo(k)fluoranthene	0.19068	0.05339	0.04989	0.01278	0.02319	0.01149	0.005	0.01469
Benzo[a]fluoranthene	0.24801	0.06944	0.06489	0.01662	0.03017	0.01495	0.00565	0.01911
Benzo[b]chrysene	0.2482	0.08062	0.06201	0.03236	0.02712	0.01412	0.00596	0.01693
Benzo[b]naphtho[2,1-d]thiophene	2.82	1.682	0.8	0.26167	0.16517	0.0842	0.0527	0.00464
Benzo[c]phenathrene	1.097	0.746	0.363	0.10833	0.08383	0.05	0.0304	0.00496
Benzo[g,h,i]fluoranthene	0.01596	0.01412	0.01038	0.00989	0.00782	0.00441	0.00134	0.00582
Benzo[j]fluoranthene	0.24598	0.06887	0.06436	0.01649	0.02992	0.01482	0.0056	0.01896
Benzothiophene	0.08433	0.06202	0.01368	0.01171	0.03658	0.00739	0.00512	0.00721
Biphenyl	71.93	51.899	20.3973	6.193	4.812	2.4515	1.5331	0.0045
C1-Benzothiophenes	48.269	19.366	8.6145	2.68933	2.2965	0.7895	0.6825	0.01586
C1-Chrysenes/Benzanthracenes	18.436	11.567	4.55775	1.78333	0.97833	0.5879	0.3698	0.0164
C1-Decalins	517.505	558.839	154.268	24.2297	135.855	13.0586	11.2507	0.10588
C1-Dibenzothiophenes	57.302	36.672	16.8288	5.8085	4.89683	0.4997	1.0731	0.01393
C1-Fluoranthenes/Pyrenes	9.635	6.131	2.4005	0.978	0.50633	0.4032	0.223	0.02413
C1-Fluorenes	286.471	183.378	76.6793	23.7957	45.7147	7.7807	4.6009	0.01076
C1-Naphthalenes	778.654	661.434	207.454	70.5127	51.425	29.7862	17.6827	0.25502
C1-Naphthobenzothiophenes	10.27	6.283	2.915	1.176	0.7265	0.3261	0.2517	0.06555
C1-Phenanthrenes/Anthracenes	394.068	250.472	105.639	34.253	20.6692	11.3038	7.9133	0.03372
C2-Benzothiophenes	68.457	0.26459	14.2465	0.04997	0.15605	0.03153	0.02184	0.03075
C2-Chrysenes/Benzanthracenes	17.982	12.272	4.611	1.87067	1.309	0.6871	0.3964	0.04834
C2-Decalins	679.783	515.956	181.535	37.0205	112.301	13.878	11.3755	0.14316
C2-Dibenzothiophenes	58.849	38.825	17.018	6.11283	4.50733	0.5752	1.1852	0.02351
C2-Fluoranthenes/Pyrenes	35.988	26.385	11.2858	3.4995	2.73233	1.1304	0.9735	0.03197
C2-Fluorenes	244.872	171.921	69.1678	20.9652	36.34	5.4406	4.3377	0.05
C2-Naphthalenes	1730.89	1089.5	428.133	67.5983	89.7313	45.0372	30.9235	0.08571
C2-Naphthobenzothiophenes	11.809	7.674	3.51275	1.41033	0.96033	0.4629	0.2756	0.02698

C2-Phenanthrenes/Anthracenes	724.102	473.472	197.669	68.2523	17.0017	23.3519	14.7427	0.05222
C3-Benzothiophenes	24.624	0.12972	5.37225	0.0245	0.813	0.01546	0.01071	0.01508
C3-Chrysenes/Benzanthracenes	6.851	6.941	2.415	2.53383	0.923	0.4472	0.2441	0.03711
C3-Decalins	345.207	183.79	78.526	17.6042	24.601	6.8703	6.2908	0.14832
C3-Dibenzothiophenes	28.357	24.611	11.7868	3.74517	0.9155	0.2905	0.6964	0.00697
C3-Fluoranthenes/Pyrenes	36.261	24.992	11.475	3.65967	2.66317	1.3549	0.898	0.01498
C3-Fluorenes	248.628	174.881	75.139	23.7478	34.3257	2.1562	4.5463	0.04754
C3-Naphthalenes	1584.44	1048.36	1157.87	130.863	83.1853	45.1932	29.4568	0.04106
C3-Naphthobenzothiophenes	5.119	4.064	1.635	0.64233	0.449	0.2354	0.1334	0.03035
C3-Phenanthrenes/Anthracenes	185.964	129.392	56.5195	18.0535	4.1095	6.8873	4.1569	0.0839
C4-Benzothiophenes	12.055	0.23117	2.48875	0.04366	0.68567	0.02755	0.01908	0.02687
C4-Chrysenes/Benzanthracenes	0.793	2.374	0.7655	1.14633	0.26767	0.1518	0.055	0.01829
C4-Decalins	598.745	290.623	150.973	32.4507	35.7607	13.6879	7.3972	0.12371
C4-Dibenzothiophenes	12.581	5.493	4.47275	1.78	0.45217	0.0912	0.2625	0.01893
C4-Fluoranthenes/Pyrenes	20.41	14.793	6.81725	2.079	1.48033	0.7076	0.5543	0.04971
C4-Naphthalenes	135.14	100.255	38.2493	16.3932	6.95133	3.5803	2.3271	0.01103
C4-Naphthobenzothiophenes	0.743	0.946	0.53925	0.1445	0.1455	0.0665	0.0323	0.01406
C4-Phenanthrenes/Anthracenes	155.846	115.606	57.0595	14.4845	3.426	6.9094	3.5269	0.08278
Carbazole	0.03711	0.03729	0.02639	0.02515	0.00656	0.01213	0.00368	0.01681
Chrysene+Triphenylene	17.099	10.468	4.5225	1.54933	0.99	0.5139	0.2976	0.00601
cis-Decalin	0.23191	0.17055	0.03762	0.03221	0.10059	0.02032	0.01408	0.01982
Dibenz(a,h)anthracene	0.5	0.5	0.125	0.08333	0.08333	0.05	0.05	0.05
Dibenz[a,c]anthracene	0.475	0.311	0.13625	0.02163	0.01813	0.00944	0.0154	0.01132
Dibenz[a,j]anthracene	0.22141	0.07191	0.05532	0.02887	0.02419	0.01259	0.00532	0.01511
Dibenzofuran	9.273	6.089	8.11175	0.64917	0.50217	0.2829	0.219	0.0036
Dibenzothiophene	11.724	7.208	3.52525	1.1475	0.7675	0.4033	0.2198	0.00473
Fluoranthene	3.575	2.104	1.36975	0.356	0.22167	0.1454	0.0643	0.00273
Fluorene	56.417	38.302	14.5573	4.39783	2.87817	1.6504	1.0248	0.01417
Indeno(1,2,3-cd)pyrene	0.10564	0.05	0.02639	0.01377	0.01154	0.00601	0.005	0.00721
Naphthalene	210.439	193.488	64.786	21.6237	15.6955	10.0294	4.8603	0.0427
Perylene	4.066	2.852	1.176	0.36867	0.2365	0.1337	0.084	0.02862
Phenanthrene	114.716	73.092	29.3078	9.58867	6.3605	3.3359	2.3532	0.03745
Picene	0.17092	0.05548	0.09725	0.02228	0.01867	0.00972	0.0041	0.01166
Pyrene	3.72	2.237	1.21325	0.37383	0.23967	0.1309	0.0723	0.0045
Retene	0.251	0.251	0.10874	0.10364	0.08194	0.04618	0.0251	0.06093
Total PAH_50	7325.55	5029.86	2727.55	575.775	450.783	215.095	142.981	0.02791

646
647
648 Supplemental Table 3. PAH concentrations in 100% HEWAF, and tested dilutions in Experiment
649 2, at beginning of exposure (t=0) and after 48 hours (t=48). All units in µg/L.
650

chemical_name	100x t0	100x t48	2.7x t0	2.7x t48	0.9x t0	0.9x t48	0.3x t0	0.3x t48
1,2-Dimethylnaphthalene	70.1740	69.2720	4.2555	1.2860	0.6290	0.4859	0.2141	0.1777
1,3+1,6-Dimethylnaphthalene	600.9890	601.7630	32.2185	10.8214	5.4624	4.5329	1.9777	1.5012
1,4,6- and 2,3,6-Trimethylnaphthalene	246.7780	233.8830	5.6061	4.7961	2.0535	2.6777	0.7749	0.6067
1,4-Dimethylnaphthalene	74.0630	105.3890	6.8383	1.9912	0.8085	0.7053	0.3541	0.2814
1,5-Dimethylnaphthalene	92.5060	92.7340	6.1127	1.7242	0.8522	0.7430	0.2956	0.2288
1,6,7 Trimethylnaphthalene	165.8450	168.5220	3.7631	3.3195	1.3613	1.6158	0.4231	0.3378
1,8-Dimethylnaphthalene	36.2810	37.7640	4.4057	0.6737	0.2960	0.4182	0.1061	0.1036
11H-Benzo(a)fluorene	4.1660	4.4660	0.1013	0.1144	0.0463	0.0757	0.0128	0.0129
11H-Benzo(b)fluorene	14.7510	13.5600	0.3769	0.3421	0.1413	0.2852	0.0442	0.0404
1-Methylanthracene	8.3420	7.7830	0.2101	0.1623	0.0741	0.1195	0.0240	0.0197
1-Methyldibenzothiophene	19.3440	19.0830	0.5415	0.4379	0.1801	0.2622	0.0567	0.0476
1-Methylfluorene	112.0380	118.7840	4.0281	2.5700	1.1234	1.3831	0.3484	0.2839
1-Methylnaphthalene	499.8680	506.9740	5.9652	9.8905	4.4446	3.0078	1.6418	1.3235

1-Methylphenanthrene	97.4050	96.1390	2.6206	2.2000	0.9095	1.3903	0.2860	0.2424
2,6+2,7-Dimethylnaphthalene	232.0150	251.4220	12.1705	3.9941	2.2053	1.3028	0.7146	0.4922
2-Methylanthracene	6.6210	7.5480	0.1602	0.1647	0.0689	0.1257	0.0248	0.0237
2-Methyldibenzothiophene	12.5620	13.2740	0.3564	0.2822	0.1265	0.1802	0.0364	0.0306
2-Methylnaphthalene	678.0370	709.5800	6.7594	13.1679	6.1219	4.2009	2.2621	1.7332
2-Methylphenanthrene	102.5820	100.1550	2.8008	2.3824	0.9758	1.5777	0.3057	0.2566
3-Methylcholanthrene	0.8430	1.0140	0.0224	0.0270	0.0094	0.0202	0.0030	0.0029
3-Methylphenanthrene	117.0160	115.4570	3.1270	2.6777	1.0687	1.7079	0.3493	0.2957
4-Methyldibenzothiophene	50.4170	49.2500	1.3536	1.1638	0.4668	0.7103	0.1530	0.1294
9,10-Dimethylanthracene	0.0305	0.0308	0.0021	0.0011	0.0002	0.0003	0.0003	0.0002
9-Methylanthracene	0.0088	0.0090	0.0006	0.0003	0.0001	0.0001	0.0001	0.0001
9-Methylphenanthrene	119.4590	117.3300	3.2388	2.6896	1.1587	1.7311	0.3559	0.3016
Acenaphthene	0.0471	0.0531	0.0091	0.0018	0.0004	0.0005	0.0004	0.0004
Acenaphthylene	17.8310	19.0160	0.7981	0.3214	0.0001	0.1981	0.0511	0.0377
Anthracene	5.5270	5.4810	0.1392	0.1245	0.0547	0.0904	0.0158	0.0133
Benzo(a)anthracene	3.5840	3.6760	0.0965	0.0936	0.0359	0.0757	0.0108	0.0098
Benzo(a)pyrene	0.5330	0.5890	0.0135	0.0123	0.0053	0.0107	0.0017	0.0013
Benzo(b)fluoranthene	2.1990	2.0080	0.0487	0.0488	0.0211	0.0378	0.0066	0.0065
Benzo(e)pyrene	3.9340	3.8310	0.0964	0.0986	0.0376	0.0836	0.0117	0.0113
Benzo(g,h,i)perylene	0.6990	0.7630	0.0164	0.0181	0.0059	0.0142	0.0022	0.0024
Benzo(k)fluoranthene	0.0500	0.0500	0.0020	0.0020	0.0005	0.0005	0.0005	0.0005
Benzo[a]fluoranthene	0.3500	0.3650	0.0095	0.0087	0.0033	0.0062	0.0011	0.0011
Benzo[b]naphtho[2,1-d]thiophene	4.7920	4.8360	0.1260	0.1156	0.0432	0.0940	0.0123	0.0122
Benzo[j]fluoranthene	0.0320	0.0320	0.0013	0.0013	0.0003	0.0003	0.0003	0.0003
Benzothiophene	0.0595	0.0667	0.3425	0.0023	0.0006	0.0008	0.0005	0.0005
Biphenyl	75.5910	81.3240	2.0001	1.4684	0.7168	0.5352	0.2391	0.1907
C1-Benzothiophenes	49.7220	48.2530	11.6560	0.6273	0.3366	0.3873	0.1271	0.0689
C1-Chrysenes/Benzanthracenes	24.6040	23.2340	0.6672	0.6235	0.2383	0.4765	0.0686	0.0642
C1-Decalins	885.2940	799.8320	6.6218	8.8735	5.3600	2.8373	2.0577	1.2206
C1-Dibenzothiophenes	101.0220	98.6400	2.7788	2.3334	0.9387	1.3878	0.2996	0.2573
C1-Fluoranthenes/Pyrenes	20.0180	18.4470	0.4873	0.5260	0.1903	0.3790	0.0564	0.0498
C1-Fluorenes	291.8070	314.7220	10.8213	6.8323	2.8435	4.1244	0.9495	0.7890
C1-Naphthalenes	1220.3350	1232.8740	11.6456	24.5752	11.3189	8.0529	4.0294	3.1474
C1-Naphthobenzothiophenes	15.0650	15.8720	0.4405	0.3950	0.1528	0.3247	0.0415	0.0390
C1-Phenanthrenes/Anthracenes	470.8320	450.5030	12.6806	10.7438	4.3674	6.7115	1.3789	1.1750
C2-Benzothiophenes	59.5080	35.4390	0.9667	1.4617	0.3929	0.7752	0.2334	0.1156
C2-Chrysenes/Benzanthracenes	23.8340	20.5250	0.6355	0.6512	0.1943	0.4215	0.0656	0.0635
C2-Decalins	840.2490	718.8850	19.1676	9.2329	5.7362	3.3795	1.9366	1.1537
C2-Dibenzothiophenes	119.1990	118.2040	3.4999	2.8169	1.2082	2.0015	0.3437	0.3112
C2-Fluoranthenes/Pyrenes	86.8550	79.8200	1.9596	1.9514	0.8689	1.6572	0.2520	0.2112
C2-Fluorenes	213.0040	223.2910	8.3444	5.0070	2.1174	3.7030	0.6548	0.5710
C2-Naphthalenes	1771.4480	1782.5400	98.1035	32.1208	16.1610	13.8418	5.7502	4.3488
C2-Naphthobenzothiophenes	17.0690	16.7060	0.5275	0.4542	0.1615	0.3600	0.0464	0.0429
C2-Phenanthrenes/Anthracenes	933.7340	911.0260	25.7468	21.4372	8.9655	16.0181	2.6545	2.3946
C3-Benzothiophenes	10.1250	8.6740	10.2089	0.1538	0.0639	0.2240	0.0250	0.0267
C3-Chrysenes/Benzanthracenes	14.9190	12.0270	0.4413	0.4109	0.1137	0.2127	0.0417	0.0410
C3-Decalins	322.7050	278.5680	26.4831	3.7410	2.1884	2.0608	0.6821	0.4362
C3-Dibenzothiophenes	77.3680	77.7340	2.3669	1.8119	0.7415	1.3554	0.2161	0.1933
C3-Fluoranthenes/Pyrenes	86.0830	80.2160	2.0578	1.9926	0.8483	1.5575	0.2312	0.2140
C3-Fluorenes	272.4970	293.5640	11.4180	6.7693	2.8694	5.2114	0.8013	0.7162
C3-Naphthalenes	1289.5650	1260.3380	35.4571	23.9075	11.0505	14.5411	4.7359	3.6666
C3-Naphthobenzothiophenes	7.3360	6.9000	0.2564	0.1926	0.0635	0.1366	0.0181	0.0182
C3-Phenanthrenes/Anthracenes	269.9950	244.0080	7.0475	5.9267	2.5078	4.3507	0.7427	0.6631
C4-Benzothiophenes	13.1540	14.3720	0.9675	0.2379	0.1008	0.2347	0.0327	0.0246
C4-Chrysenes/Benzanthracenes	5.2240	5.5790	0.1607	0.1556	0.0394	0.0755	0.0132	0.0193
C4-Decalins	376.1180	345.7150	103.2344	5.7496	3.2116	2.8019	1.0458	0.6632
C4-Dibenzothiophenes	32.8860	38.6410	1.1247	0.8941	0.4018	0.6403	0.1027	0.0995

C4-Fluoranthenes/Pyrenes	47.4500	47.9610	1.1193	1.2299	0.5058	0.9123	0.1282	0.1178
C4-Naphthalenes	160.0960	162.6730	5.1734	4.0156	1.4164	2.6411	0.5261	0.3756
C4-Naphthobenzothiophenes	3.5570	2.9210	0.1185	0.0792	0.0261	0.0503	0.0081	0.0093
C4-Phenanthrenes/Anthracenes	242.7460	173.3800	4.9715	5.0254	2.1779	3.8735	0.5955	0.5033
Carbazole	0.0100	0.0100	0.0005	0.0004	0.0001	0.0001	0.0001	0.0001
Chrysene+Triphenylene	22.0250	22.0690	0.5609	0.5404	0.2097	0.4288	0.0645	0.0590
cis-Decalin	0.1738	0.1951	1.0010	0.0068	0.0016	0.0022	0.0015	0.0014
Dibenz(a,h)anthracene	0.0500	0.0500	0.0020	0.0020	0.0005	0.0005	0.0005	0.0005
Dibenzofuran	10.2490	10.3160	0.1848	0.2244	0.0913	0.0760	0.0370	0.0291
Dibenzothiophene	20.8200	20.5110	0.5117	0.4588	0.2016	0.2083	0.0658	0.0542
Fluoranthene	4.3050	4.1360	0.1797	0.1095	0.0421	0.0798	0.0136	0.0122
Fluorene	60.4840	56.8220	1.5207	1.2368	0.5397	0.4805	0.1878	0.1526
Indeno(1,2,3-cd)pyrene	0.0500	0.0500	0.0020	0.0020	0.0005	0.0005	0.0005	0.0005
Naphthalene	422.8900	440.0770	5.0519	8.7646	3.7109	2.6882	1.3626	1.0925
Perylene	0.1460	0.2120	0.0020	0.0044	0.0014	0.0023	0.0006	0.0006
Phenanthrene	129.6710	124.9130	3.2668	2.7960	1.1434	1.2940	0.4021	0.3266
Pyrene	5.4050	5.2520	0.1286	0.1263	0.0532	0.0909	0.0170	0.0151
Retene	0.0504	0.0510	0.0033	0.0020	0.0004	0.0005	0.0005	0.0004
Total PAH_50	8609.4370	8518.3310	264.8012	179.4458	79.4047	101.5104	27.2542	22.1295