Discriminating source of oil contamination in teleost fish, *Lates calcarifer*, using multivariate analysis of a suite of physiological and behavioural biomarkers

Francis Spilsbury[a]*, Alan Scarlett[b], Kliti Grice[b], Marthe Monique Gagnon[a]

* Corresponding author

[a] School of Molecular and Life Sciences, Curtin University, Kent Street, Bentley, Western Australia 6102

[b] Western Australian Organic and Isotope Geochemistry Centre, The Institute for Geoscience Research, School of Earth and Planetary Science, Curtin University, GPO BOX U1987, Perth, WA 6845, Western Australia

Keywords

Crude oil, biomarkers, barramundi, bunker C, Montara, PCA

Highlights:

- Fish exposed to crude and heavy fuel oils via dietary exposure for 33 days
- Distinctive profiles of 12 biomarkers produced for oil-exposed fish
- Individual biomarker responses dependent on characteristics of exposure oil
- PCA analyses able to discriminate between crude and heavy fuel oil exposure
- Biomarker profiles inform on the oil characteristics biota is exposed to

Abstract

The release of petroleum hydrocarbons into the environment from natural seeps, well blowouts, pipeline leaks, shipping accidents and deliberate tank washing poses an ongoing threat to marine ecosystems. Distinguishing the source of oil contamination in exposed biota can be relatively straightforward if samples of the oil are available but, in their absence, such discrimination in fish poses a major challenge. The use of physiological and behavioural biomarker analysis provides a useful tool to describe sub-lethal effects of toxicant exposure.

In this study we describe the responses of 12 biomarkers in *Lates calcarifer* (Asian seabass) following a 33-day dietary exposure (1%w/w) to heavy fuel oil (HFO) and to Montara, a typical Australian medium crude oil (MCO). Principal components analysis was used to differentiate between fish exposed to HFO from those exposed to MCO. Inferences can be made about the composition of an oil from the biomarker profiles produced in exposed fish.

Introduction

The introduction of petroleum hydrocarbons into the environment can occur from natural processes such as marine seeps (Burns *et al.*, 2010) or due to human activities. Large-scale anthropogenic releases such as the Deepwater Horizon oil spill are extreme events with long-term environmental consequences (Nunes *et al.*, 2015; Snyder *et al.*, 2017; Smeltz *et al.*, 2017). Although on smaller scale, the blowout from the West Atlas (Montara) well platform in Northwestern Australia in 2009 resulted in the unintentional release of an estimated 4,750 tonnes of medium-light crude oil (Gagnon and Rawson, 2012; Burns and Jones, 2016; Spies *et al.,* 2017) over a period of 74 days (Hunter, 2010). Shipping accidents periodically occur, resulting in highly publicized released of petroleum hydrocarbons into the environment such as the 2002 Prestige spill of 17,000 tonnes of heavy fuel oil (HFO) off the coast of Spain (Albaigés Riera *et al.*, 2006; Gonzales *et al.*, 2006), and the recent grounding of the *M.V.* Wakashio in Mauritius in 2020 where an estimated 1000 tonnes of a new type of low sulphur fuel oil was spilled (Seveso *et al.*, 2021).

Crude oils are highly complex mixtures of several thousand compounds with chemical biomarker profiles that differ greatly depending on the source. In heavily developed industrial areas, petroleum hydrocarbon pollutants found in the environment may originate from several sources, each with a distinctive chemical fingerprint (Elfadly *et al.*, 2017). Heavy fuel oil (HFO) (also termed bunker oil or heavy diesel oil) refers to blended residual products from the distillation of crude oil commonly used in merchant vessels (Fritt-Rasmussen *et al.*, 2018). HFO produced from different crude oils are distinguishable by specific chemical biomarkers (Uhler *et al.*, 2016). Their universal use in shipping

53 has led to their frequent release either intentionally (e.g. tank washing) or accidentally, and hence
54 an understanding of the environmental effects of HFO discharges is important. On exposure to the
55 environment, the composition of crude and fuel oils changes rapidly as lower molecular weight
56 volatile compounds evaporate, water-soluble compounds enter the water column, microbial
57 metabolism and UV-degradation all combine to weather crude oil until eventually the asphaltene-
58 rich residue fraction remains, often washing up on beaches as tar balls (Scarlett *et al.*, 2019).

59 Laboratory-based ecotoxicological studies seek to simulate a complicated environmental picture
60 where sub-lethal effects play a significant role (Whitehead, 2013) in the impacts to organisms in a
61 spill-affected area. The various biomarkers measured in such studies can show evidence of exposure
62 to a class of toxicants, or provide quantitation of the effects of this exposure (van der Oost *et al.*,
63 1993). Many previous toxicity studies have concentrated on the Water-Accommodated Fraction
64 (WAF) of crude oil, and have sought to simulate the complex, partition-driven adverse
65 environmental effects of oil spills by using flow-through systems over contaminated gravel (e.g.
66 Heintz *et al.*, 1999) or mechanical methods (e.g. Aas *et al.*, 2000) to generate WAF from crude oils.
67 Laboratory methods to generate WAF often result in highly variable concentrations of the
68 compounds of interest which makes replication difficult (Singer *et al.*, 2000; Barron *et al.*, 2003).
69 Studies using dietary exposures are possibly more repeatable, but there is limited data available.
70 Exploring the sub-lethal toxigenic effects of crude oil compounds via the dietary route has shown
71 behavioral changes in Siamese fighting fish (*Betta splendends*) (Bautista *et al.*, 2019) and zebrafish
72 (*Dario rario*) (Vignet *et al.*, 2014b), activation of Cyp1a mediated responses (Narghang *et al.*, 2010)
73 and changes in serum biochemistry (Vieweg *et al.*, 2018) in polar cod (*Boreogadus saida*), and
74 growth inhibition in zebrafish (Vignet *et al.*, 2014a). Hence, dietary exposure has the potential to
75 produce reproducible sublethal effects using well-characterised whole oils.

76 Fish present in a spill-affected site may be exposed to toxicants from crude oils via dietary intake, or
77 water-borne via the gills. Various species of fish from sites with high sediment petroleum

78 hydrocarbon concentrations show absorption and retention of crude oil compounds in muscle tissue
79 (Ahmed *et al.*, 2019). Lipophilic compounds (i.e. with an octanol-water partition coefficient $LogK_{OW}$ >
80 4) have previously been shown to be taken up by fish via the dietary route (McKim, 1994; Law and
81 Hellou, 1999), but there is a paucity of data on this. Anecdotally, the authors have observed fish in
82 oil spill affected areas feeding on floating wax residues coated with oil, mistaking them for food.
83 Bioconcentration and biomagnification may enhance the impacts of crude oil toxicogenuic
84 compounds to marine organisms (Varanasi, 1989; Hellou *et al.*, 2004). Compounds with $LogK_{OW}$ >
85 4.5 are likely to bioaccumulate (Veith *et al.*, 1979; Hellou *et al.* 2002; Lombardo *et al.,* 2010; Gissi *et*
86 *al.*, 2015; ECHA, 2017) and biomagnify in food webs (Voutsas *et al.,* 2002).

87 The classical toxicogenesis of petrogenic compounds such as PAHs has been well-described
88 elsewhere (reviewed by Renaud and Deschaux, 2006). Likewise, the adverse effects of metals on fish
89 physiology and behavior are well-established (Atchison *et al.*, 1987; Wood, 2011). However, given
90 the enormous number of compounds present in crude oils, it is exceedingly difficult to describe the
91 toxic effects of the individual constituent compounds contributing to observed adverse effects.
92 Hence to fully describe the toxicity of a crude oil, it is necessary to study its effects *in toto*, rather
93 than selectively choosing groups of known toxicogenic compounds and applying classic mixture
94 toxicity models.

95 *Lates calcarifer,* commonly known as Asian seabass, barramundi or Australian seabass is a predatory
96 teleost fish found in both freshwater, estuarine and marine environments. A popular sportsfish and
97 important for aquaculture (Mathew, 2019), it is raised in commercial operations throughout south-
98 east Asia (Boonyaratpalin, 2017) and elsewhere (Hardin and Hill, 2012). It has a wide global natural
99 distribution in temperate and tropical waters with genetically distinct natural populations (Yue *et al.*,
100 2009) ranging from the eastern tip of Papua New Guinea to the Persian Gulf (Grey, 1987). Its wide
101 distribution and hardy ability to tolerate a range of environmental conditions make it a suitable test
102 species for laboratory-based studies concerned with the ecotoxicological effects of crude oil spills.

103 Following an oil spill, the ability to distinguish whether fish have been exposed to a medium crude oil
104 or a heavy fuel oil could be of benefit in terms of assessing the impact on ecosystem health and
105 litigation proceedings. In this study, we aim to ascertain if exposure to two different petroleum
106 products, a heavy fuel oil and a medium crude oil, produce significantly distinct effects in a common
107 teleost fish. In addition to individual biomarker responses, we aim to establish if the integrated set
108 of biomarkers has the potential to discriminate between the biomarker responses in such a way as
109 could be predicted based on the character of the oils. Overall, we aim to test the hypothesis that the
110 source of the binary exposure could be differentiated based on a suite of physiological and
111 behavioral biomarkers as measured in *L. calcarifer*.

112

113 Materials and Methods

114 Characterization of Oils

115 The HFO, a typical bunker C fuel oil (API 11.4) was supplied by the BP Kwinana Oil Refinery (Western
116 Australia). The Australian MCO (API 31.0) was supplied by PTTEP Pty Ltd. As highly volatile
117 components within oils are usually rapidly lost on exposure to the environment, the medium crude
118 oil (MCO) was weathered for 5 days using a published method (Smith *et al.*, 2006) to simulate post-
119 spill conditions. Heavy fuel oils are typically blends of residual post-refinement products (Lewis,
120 2002; Fritt-Rasmussen *et al.*, 2018), and have already undergone treatments exceeding the
121 weathering protocol used for MCO. The HFO was analysed as received.

122 Metals Analysis

123 A sample of crude oil was accurately weighed and repeatedly digested in nitric acid, and finally in a
124 mixture of nitric/perchloric acids. The digestate was taken to incipient dryness and the residue was
125 dissolved in high purity nitric (0.7 mL), hydrochloric (0.2 mL) acids and high purity water (25 mL).
126 Samples were analysed in triplicate, and quantified by inductively coupled plasma atomic emission

127 spectroscopy (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS) against a
128 commercial standard (AccuTrace High Purity multi-element standards, Choice Analytical).

129

130 ## Preparation of Spiked Fish Feeds

131 Dry fishmeal (Nova FF 3mm, Skretting Pty Ltd, Perth, Australia) was powderized in a food processor,
132 and then 180g samples were spiked with either 3.4 g of HFO or weathered MCO and mixed
133 thoroughly in a stainless steel benchtop mixer before 200 mL of warmed 10% w/v gelatin solution
134 was added. The mixture was uniformly spread on a stainless steel tray, covered in aluminum foil and
135 placed in an air-tight container at 4°C for 12 h. On setting, the resultant fish feed was manually sliced
136 into approximately 2 mm cubes, weighed and stored at -20°C until used.

137 All stainless steel mixing and cutting apparatus was thoroughly cleaned, and double-rinsed with
138 methanol followed by dichloromethane (DCM) between preparations.

139

140 ## Polycyclic Aromatic Hydrocarbons

141 The MCO, HFO and fish feeds spiked with the respective oils were analysed for a suite of 40 PAHs
142 using standard published methods (Forth *et al.*, 2017). Oils were diluted in DCM, and an internal
143 standard added to a 1ml aliquot of the extract. Fish feeds (10g) were extracted by sonication in
144 acetone/DCM, and chemically dried using sodium sulphate.

145 Oils and fish feed extracts were analysed for PAHs using GC mass spectrometry (GC-MS) selected ion
146 monitoring (SIM). PAHs were quantitated by comparison to external standards (Accustandard,
147 Connecticut, U.S.A.). Alkylated-PAHs were quantitated using the response factors of the appropriate
148 parent PAH using the protocol of Forth *et al*. (2017). All extractions and analyses were performed in
149 triplicate.

150

151 ## Fish Exposure and Sampling

152 Juvenile barramundi (10-15 cm) were obtained from a commercial hatchery. Originally raised in
153 freshwater, the fish were gradually acclimatized to 34 ppt salinity seawater over 5 days before being
154 transferred to 100 L tanks. Natural Indian Ocean seawater was collected from a coastal region 100
155 km north of Perth, Western Australia. Fish were handled and maintained in accordance with Curtin
156 University animal ethics approval ARE2019/11. Each of the three exposure groups (negative control,
157 MCO and HFO) were tested in triplicate, with 4 fish per tank (n=12 per treatment). A closed
158 recirculating system via an external canister biofilter was used with a flow rate of approximately
159 5L/min. Water was maintained at 28 ^{o}C (±2 ^{o}C) using in-tank submersible heaters, and was aerated
160 to achieve dissolved oxygen of not less than 5.0 mg/L. Fish health was maintained by daily
161 monitoring of total ammonia, dissolved oxygen, pH, salinity and temperature with partial water
162 exchanges of 10 - 60% of the 100 L tank volume performed daily as required.

163 Fish were fed twice per day to a total of 2% body weight per day, with either commercial fish meal
164 (negative control) or commercial fish meal spiked with 1 % w/w MCO or 1 %w/w HFO. Post-feeding,
165 excrement and any fish feed not consumed was removed one hour after feeding.

166 Fish were exposed for 33 days, followed by 2 days without feeding to ensure sufficient contents of
167 the bile duct for sampling. Fish were euthanized by ike-jime, a blood sample was immediately taken
168 from the caudal vein using an un-heparinized syringe. Haematocrit was measured by the capillary
169 method using heparinized tubes, and blood was allowed to clot for 45 minutes on ice before
170 centrifugation at 5000xG for 5 minutes followed by removal of serum into 2 mL cryovials which were
171 snap frozen in liquid nitrogen before being stored at -80 ^{o}C until analysis. Physiological parameters of
172 standard and fork length, whole wet weight and carcass weight (body weight without viscera) were
173 recorded.

174 The liver was excised and weighed, the brain was surgically removed, samples of gill tissue were
175 excised, and bile was collected directly from the bile duct using a 1.0 mL syringe and 22-gauge
176 needle. All tissue samples were divided among several separate 2 mL cryovials which were
177 immediately snap frozen in liquid nitrogen and stored at -80°C until analysis.

178

179 ## Physiological Parameters

180 Fulton's condition factor (CF) was calculated as:

181 $$\text{CF} = \left[\frac{W_c}{{L_f}^3}\right] \times 10^6$$

182 where L_f is the fork length (in mm) of the fish and W_c is the carcass weight (g).

183 The hepatosomatic index (HSI) was calculated as:

184 $$\text{HSI} = \left[\frac{W_l}{W_c}\right] \times 100$$

185 where W_c is the carcass weight (g) and W_l is the liver weight (g).

186

187 ## Biochemical Analyses

188 DNA damage was estimated by quantifying 8-oxo-dG in serum using a commercially available ELISA
189 kit (StressMarq Biosciences, Vancouver, Canada, catalog number SKT-120-965) as per
190 manufacturer's instructions.

191 Acetylcholinetserase (AChE) in brain tissue was quantified using a commercially available ELISA kit
192 (Cusabio Biotech, Houston, U.S.A., catalog number CSB-E17001Fh). Samples were thawed on ice,
193 surface rinsed with chilled phosphate buffered saline, pH 7.4 (PBS) and a 10% w/v homogenate
194 prepared in PBS. Samples were not diluted prior to analysis.

195 Heat shock protein 70 (HSP70) in gill tissue was similarly quantified using a commercially available
196 ELISA kit (Cusabio Biotech, Houston, U.S.A., catalog number CSB-E16327Fh). Samples were thawed
197 on ice, surface rinsed with PBS, and a 10% w/v homogenate of excised lamellae prepared in PBS.

198 Ethoxyresorufin deethylase (EROD) activity was quantified in liver tissue using a published
199 spectrofluorimetric method (Hodson *et al.*, 1991). Liver samples were thawed on ice and a 20% w/v
200 homogenate prepared in chilled HEPES buffer, pH 7.5. Homogenates were centrifuged at 12000xg
201 for 20 minutes at 4°C, and the microsome-rich S9 fraction of the supernatant was collected for
202 analysis. EROD activity was reported as pmol of substrate converted to product per minute.

203 Biliary PAH metabolites were estimated using the method of Lin *et al.,* 1996. As standards, naphthol
204 (excitation/emission wavelengths of 290/335nm), a phenanthrol standard (Torreira-Melo, 2015)
205 (excitation/emission wavelengths of 260/380nm) and pyrenol (excitation/emission wavelengths of
206 340/380nm and 380/430nm for pyrene-type and benzo(*a*)pyrene-type metabolites respectively)
207 were used. Sample fluorescence was measured using a Perkin–Elmer LS-5 Luminescence
208 Spectrometer, and reported as μg of equivalent fluorescence of the relevant standard-type.

209 All biochemical biomarkers were normalised to total protein in the sample, measured using the
210 Bradford method (Bradford, 1976; Bio-Rad, 1979) with bovine serum albumin (BSA) as a standard
211 and a BioRad iMark Microplate Absorbance Reader to measure absorbance at 595nm.

212

213 Behavioural Effects

214 Impacts on foraging behaviour was estimated via the rate of food consumption. Daily feed was
215 weighed, and the time taken for each tank of four fish to consume their allotment of approximately
216 5g of food was measured and averaged by the number of fish in the tank (i.e. 4 fish). Feeding rate
217 was reported in grams of food ingested per minute per fish (g/min/fish).

218

219 ## Liver Histomorphology

220 Four liver samples from each treatment group were randomly selected for histomorphological
221 analysis. Samples were sectioned, mounted and stained by the Western Australian Government
222 Department of Primary Industries and Regional Development (DPIRD) and interpreted by a
223 veterinary pathologist.

224

225 ## Data Handling

226 All data analyses were conducted using R statistical software, version 4.02. Significant difference
227 between means of exposure groups for the various biomarkers was established using Tukey's HSD.
228 Differences in biomarker profiles between exposure groups were characterised by principal
229 components analysis (PCA) (Le *et al.*, 2008). Individuals missing values for any particular biomarker
230 were included in the PCA analysis by substituting missing values with the mean of the respective
231 exposure group for that biomarker (Husson *et al.*, 2016).

232

233 # Results and Discussion

234 All confidence intervals provided are standard error.

235 ## Characterization of Oils

236 The HFO was found to be highly sulfurous (10200 ± 5900 mg sulphur/kg), with higher levels of iron
237 (37.90 ± 21.8 mg/kg), nickel (12.23 ± 7.06 mg/kg) and vanadium (15.27 ± 8.81 mg/kg) relative to
238 MCO but differences between other element concentrations were less pronounced (Table 1).

239 MCO contained higher concentrations of naphthalenes (29800 ± 1180 mg/kg) and phenanthrenes
240 (6370 ± 210 mg/kg) than the HFO (11900 ± 124 mg/kg and 4830 ± 39 mg/kg respectively).
241 Conversely, the HFO contained higher concentrations of the larger 4-ring pyrenes (2550 ± 49 mg/kg)

242 than MCO (910 ± 22 mg/kg). Of particular ecotoxicological interest, the HFO contained 891 ± 29
243 mg/kg benzopyrenes, which were absent in MCO.

244 Total PAH concentration (a sum of 40 measured PAH compounds) measured in fish feed used in this
245 study averaged 600 mg/kg (MCO) and 425 mg/kg (HFO) fish food respectively (Table S1. These are
246 environmentally relevant concentrations: in spill-affected zones after the Deepwater Horizon
247 incident, total PAH concentrations in sediments were as high as 355mg/kg (Turner *et al.*, 2014) and
248 856mg/kg (Wang *et al.*, 2014).

249

250 Physiological Parameters

251 Mean CF was significantly lower ($p = 0.015$) in HFO exposed fish (14.38 ± 0.44) compared to negative
252 controls (16.09 ± 0.25) (Figure 1a). Mean CF in MCO (14.73 ± 0.48) exposed fish were also
253 comparably lower than negative control fish, but not significantly so ($p = 0.060$). Toxicant exposure
254 carries with it an associated energy burden on the organism (Marchand *et al.*, 2004) as it both
255 metabolizes and excretes xenobiotic compounds, and repairs any associated damage that may
256 occur, for example by reactive oxidative species (ROS) generated through the Cyp1a mediated
257 metabolism of PAHs. The CF of fish exposed to MCO was not significantly different from that of fish
258 exposed to HFO ($p = 0.819$), suggesting that the specific composition of the oil does not affect the
259 energy burden required by the organism to deal with ingested toxicants.

260 Hepatosomatic Index was not significantly different between any of the treatment groups ($p =$
261 0.093) (Figure 2b). Faster growing juvenile fish tend to show higher rates of liver hyperplasia than
262 slower growing adult fish (van der Oost *et al.,* 2002), but as the liver has both storage and
263 detoxifying functions, the enlargement of the liver in response to exposure to a toxicant can be
264 reduced to the point of no-net increase by poor nutrition (Schlenk and Benson, 2017). It may also be
265 that the duration of our trial at 35 days was insufficient for an increase to be seen in the liver size of
266 fish exposed to petroleum hydrocarbons. Significant HSI responses to hydrocarbons from crude oil

267 were not found in other laboratory exposure studies in Atlantic cod (*Gadus morhua*) (Aas *et al.*,
268 2000) or Atlantic salmon (*Salmo salar)* (Gagnon and Holdway, 2002). Field studies following the 2009
269 Montara oil spill similarly showed no significant changes to HSI (Gagnon and Rawson, 2012) in either
270 red emperor (*Lutjanus sebae*) or goldband snapper (*Pristipomoides multidens*) despite the 74-day
271 duration of the petroleum release (Hunter, 2010, Burns *et al.* 2010).

272 Haematocrit varied between treatment groups (Figure 1c). HFO exposed fish had a significantly
273 lower ($p = 0.001$) mean haematocrit (0.199 ± 0.014) compared to negative control fish (0.290 ±
274 0.018). Repeating the pattern found with CF, MCO-exposed fish also had a lower haematocrit (0.238
275 ± 0.012) which approached significance ($p = 0.069$). Lower haematocrit implies a reduction in blood
276 oxygenation, which has metabolic consequences that are possibly a contributing factor in the lower
277 CF evident in fish exposed to crude oils.

278

279 ## Biomarkers of Exposure:

280 Biliary PAH metabolites in each treatment varied generally proportionately to the relative
281 abundance of the parent compounds in the respective crude oils (Figure 1(i), (j), (k) and (l)). Mean
282 biliary metabolite concentrations in MCO- and HFO-exposed fish were significantly different from
283 negative control fish ($p < 0.001$), with the exception of benzo(*a)*pyrene type metabolites in MCO-
284 exposed fish (15.96 ng/mg protein ± 0.67) which were non-significantly higher ($p = 0.120$) than
285 negative control fish (7.69 ng/mg protein ± 0.39), reflecting the paucity of larger molecular weight
286 PAHs found in the MCO used in this study.

287 Compared to negative controls, EROD activity in fish exposed to MCO showed no significant increase
288 compared to negative controls ($p = 0.995$). EROD activity was clearly induced in fish exposed to HFO
289 (2.08 ± 0.39 pmol/min/mg protein), which was significantly higher than both the negative control
290 group (0.96 ± 0.08 pmol/min/mg protein, $p = 0.012$) and fish exposed to MCO (0.99 ± 0.18
291 pmol/min/mg protein, $p = 0.026$) (Figure 1f).

292 Although the ability of petroleum hydrocarbons to induce EROD activity varies greatly between fish
293 species (White *et al.*, 2000), *L.calcarifer* exhibits significant EROD induction following intra-peritoneal
294 injection of petroleum oils (Mercurio *et al.*, 2004; Gagnon and Rawson, 2017). Lower molecular
295 weight PAHs such as naphthalenes (two rings) and phenanthrenes (three rings) that are present in
296 relatively high abundance in MCO have a lower CYP1a induction potential than the larger PAHs with
297 four or five ring structures (Whyte *et al.*, 2000). The lack of EROD induction in MCO-exposed fish is
298 likely due to the paucity of higher molecular weight compounds in the PAH profile of MCO.

299 Gill tissue HSP70 concentration was elevated in HFO-exposed fish (4.71 ± 0.64 pg/mg protein) and in
300 MCO-exposed fish (4.86 ± 0.51 pg/mg protein) compared to negative control fish (3.87 ± 0.38 pg/mg
301 protein) (Figure 1g), but this was not statistically significant (t-test, $p \geq 0.10$). HSP70 induction is a
302 complex biological process (Morimoto, 1998), is not specific to petroleum hydrocarbons (Whyte *et*
303 *al.*, 2000), and can be induced by several classes of compounds (e.g. PCBs).

304

305 ## Biomarkers of Effect

306 The levels of DNA damage (as 8-oxo-dG) did not change between test groups, with serum
307 concentrations of 0.880 ± 0.047 ng/mg protein, 0.889 ± 0.043 ng/mg protein and 0.850 ± 0.039
308 ng/mg protein detected in negative control, MCO- and HFO-exposed fish respectively. PAHs and
309 metals are among the causes of elevated serum 8-oxo-dG (Valvanidis *et al.*, 2009). The bioavailability
310 of metals is a crucial factor in the mechanism of oxidative DNA damage from coal fly ash, rich in
311 vanadium and nickel (Prahalad *et al.*, 2000). Although vanadium and nickel are present in HFO in
312 small amounts (15.3 μg/g and 12.2 μg/g respectively), metals in crude oils are generally found in the
313 asphaltene fraction complexed inside porphyrins (Biesaga *et al.*, 2000) and other metal porphyrins
314 can exist (Woltering *et al.*, 2016), and may not be bioavailable via the dietary route. This could
315 explain the observed lack of difference in 8-oxo-dG between groups in our experiment.

316 AChE concentration in brain tissue homogenate decreased significantly (t-test, $p \leq 0.025$) in fish
317 exposed to HFO (0.69 ± 0.05 ng/mg protein) and MCO (0.86 ± 0.05 ng/mg protein) compared to fish
318 in the negative control group (0.90 ± 0.04 ng/mg protein) (Figure 1h).

319

320 Behavioral Changes

321 *L. calcarifer* are a known sportfish, and aggressively compete for food even in captivity. Fish in the
322 negative control group had a mean feeding rate of 4.51 ± 0.10 g of food ingested/min/fish. Fish
323 exposed to petroleum hydrocarbons exhibited significantly lower ($p < 0.001$) feeding rates of 2.17 ±
324 0.09 g/min/fish and 0.244 ± 0.01 g/min/fish for MCO- and HFO-exposed fish respectively (Figure 1d).

325 Anecdotally, fish exposed to HFO visually appeared intoxicated, slow swimming or immobile, and
326 were slow to respond to stimuli. Similar observations have been reported in other fish species
327 exposed to petroleum hydrocarbons (reviewed by Kasumayan, 2001; Weiss and Candelmo, 2012).
328 Exposure to the WAF of fuel oils was reported to impair the ability of rainbow trout (*Oncorhynchus*
329 *mykiss*) to successfully predate (Folmar *et al.*, 1982), and greatly reduced the feeding rate of gobies
330 (*Gobionellus boleosoma) (Greg et al.*, 1997). The present study demonstrates that dietary exposure
331 also produces typical narcosis effects in barramundi.

332 Among the various drivers of adverse behavioral impacts in fish, cholinesterase inhibition is an
333 important mechanism driving behavioral pathology (Scott and Sloman, 2004). In the present study,
334 an association appears to be present between lowered AChE and decreased feeding rate. This agrees
335 with findings in other studies that suggest lowered AChE activity in response to toxicant exposure in
336 mosquitofish (*Gambusia affinis*) is associated with decreased swimming speed (Rao *et al.*, 2005). In a
337 laboratory setting, exposure to phenanthrene has been shown to cause reduced swimming speed
338 and alter swimming patterns in guppies (*Poecilia vivipara)* (Torreira-Melo *et al.*, 2015). In the field,
339 brown trout (*Salmo trutta)* swim slower in streams highly polluted with a complex mixture of
340 toxicants including PAHs than in more mildly polluted streams (Triebskorn *et al.*, 1997).

341 There also appears to be a relationship in the present study between decreased haematocrit and
342 reduced feeding rates. This agrees with other findings that decreased haematocrit and red blood cell
343 count is associated with decreased swimming speed and predation activity in *L. calcarifer*
344 (Satheeshkumar *et al., 2012)*, providing a second measure of a biological impact which might
345 translate into reduced foraging ability in PAH-exposed fish.

346

347 Liver Histomorphology

348 Histomorphological analysis showed only very minor qualitative differences between test groups (Fig
349 S1). Adipocytes were generally plump and clear in appearance, except for MCO-exposed fish which
350 were mildly collapsed. Hepatocytes were slightly smaller in size in MCO- and HFO-exposed fish
351 compared to negative control fish. In both MCO and HFO test groups, zymogen granules were
352 observed in 50% of the exocrine pancreas cytoplasm, compared to 50-70% in the negative control
353 group.

354 If dietary exposure to crude oils caused hyperplasia (i.e. enlarged hepatocytes), a higher HIS would
355 be expected, however in the current study the lack of variation in hepatocyte size was mirrored by
356 the lack of variation in HSI. General indications of long-term toxicant exposure include toxicopathic
357 liver lesions, and elevated macrophage immigration and the resulting macrophage aggregates (Guilio
358 and Hilton, 2008). Toxicopathic liver lesions in English sole (*Pleuronectes vetulus)* were associated in
359 a dose-dependent manner with sediment PAH concentrations and biliary PAH metabolites in a field
360 survey of the Vancouver Harbour, Canada (Stehr *et al.*, 2004). It is possible that the trial exposure
361 duration of 33 days was insufficient to cause significant histological changes.

362

363 ## Biomarker Baseline

364 The normal, or baseline, ranges for the measured suite of biomarkers, in healthy *L. calcarifer* not
365 exposed to oils are presented in Table 2. The baseline ranges were defined as 2× the standard error
366 of the mean value from the negative control group (OSPAR, 2013). Data on pre-exposure values for
367 biomarkers are of critical importance in environmental impact studies attempting to estimate the
368 adverse effects of an oil spill (Nunes *et al.,* 2015). In the aftermath of the Deepwater Horizon oil spill,
369 the absence of pre-incident baseline data of fish health was an obstacle to fully assessing the long-
370 term environmental impacts of the incident (e.g. Shigenaka, 2014; Murawski *et al.*, 2014).

371

372 ## Multivariate Analysis

373 A PCA of 11 of the biomarkers included in the study using Bray-Curtis distancing shows two principal
374 components which represent 50.7% of the total variability of the combined biomarker dataset
375 (Figure 2).

376 The three treatment groups are significantly separated from each other (Tukey's HSD, $p < 0.05$), and
377 their positions on the principal component axes are driven by different biomarkers. The positions of
378 HFO-exposed fish are influenced by the presence of pyrene-type and benzo(a)pyrene type biliary
379 metabolites, increased EROD activity, and lower AChE in brain tissue. MCO-exposed fish are
380 influenced by naphthalene-type and phenanthrene type biliary metabolites, and by HSP70
381 concentration in gill tissue. The position of negative control fish is largely determined by higher
382 condition factor and haematocrit, and an absence of other elevated biomarkers.

383 Biliary PAH metabolites, AChE concentration and EROD activation had the highest discriminatory
384 power in describing the exposure and effects of petroleum hydrocarbon exposure in *L. calcarifer.*
385 DNA damage (as serum 8-oxo-dG), HSI and HSP70 had the least. This agrees with findings in similar
386 dietary petroleum hydrocarbon exposure studies in other species (Nahrang *et al. 2009).*

The separation on the principal component axes is in accordance with the respective composition of the crude oils to which the fish were exposed. MCO has higher concentrations of naphthalenes and phenanthrenes and virtually no pyrenes or benzo(*a*)pyrenes, implying that it will be a poor inducer of Cyp1a enzymes such as EROD. In contrast, HFO has relatively low concentrations of two and three ring aromatics and greater concentrations of larger structures. In the absence of actual chemical analyses of an oil, general inferences can be made about the composition of the crude oils to which *L. calcarifer* were exposed, given the signature differences in biomarker responses of exposed fish. Following an oil spill it can be assumed that fish ill-health is related to the oil known to be spilled, but it is possible that fish have been exposed to a different petroleum hydrocarbon source or other stressors. The integration of all biomarkers in a single PCA biplot may help to confirm or reject certain oils as sources for observed adverse effects on fish in an oil spill zone, and reinforce evidence that fish have been exposed to, and affected by, exposure to petroleum hydrocarbons.

Acknowledgements

Many thanks to Professor Steve Rowland for advice and insight in the writing of this manuscript, and to Rowan Kliendienst for trial exposure tank set up and sampling assistance.

Funding

This work was supported by grants provided by the Australian Research Council (grant number LP170101000).

References

Aas, E., Baussant, T., Balk, L., Liewenborg, B. and Andersen, O.K., 2000. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod. *Aquatic Toxicology*, *51*(2), pp.241-258.

Ahmed, O.E., Eldesoky, A.M. and El Nady, M.M., 2019. Evaluation of petroleum hydrocarbons and its impact on organic matters of living organisms in the northwestern Gulf of Suez, Egypt. *Petroleum Science and Technology*, 37(24), pp.2441-2449.

Albaigés Riera, J., Morales-Nin, B. and Vilas, F., 2006. The Prestige oil spill: a scientific response.

Atchison, G.J., Henry, M.G. and Sandheinrich, M.B., 1987. Effects of metals on fish behavior: a review. *Environmental Biology of Fishes*, *18*(1), pp.11-25.

Barron, M.G. and Ka'aihue, L., 2003. Critical evaluation of CROSERF test methods for oil dispersant toxicity testing under subarctic conditions. *Marine Pollution Bulletin*, *46*(9), pp.1191-1199.

Bautista, N.M., Pothini, T., Meng, K. and Burggren, W.W., 2019. Behavioral consequences of dietary exposure to crude oil extracts in the Siamese fighting fish (*Betta splendens*). *Aquatic Toxicology*, *207*, pp.34-42.

Biesaga, M., Pyrzyńska, K. and Trojanowicz, M., 2000. Porphyrins in analytical chemistry. A review. *Talanta*, *51*(2), pp.209-224.

Bio-Rad, 1979. Protein Assay Instruction Manual, "Bio-Rad Laboratories." *Richmond, CA* (1979): 1-16.

Bradford, M.M., 1976. A sensitive method for the total protein determination using the principle of protein-dye binding. *Analytical Biochemistry* , 72, pp.249-251.

Boonyaratpalin, M., 2017. Asian seabass, *Lates calcarifer*. In Handbook of Nutrient Requirements of finfish (pp. 5-12). CRC Press.

Burns, K.A., Brinkman, D.L., Brunskill, G.J., Logan, G.A., Volk, H., Wasmund, K. and Zagorskis, I., 2010. Fluxes and fate of petroleum hydrocarbons in the Timor Sea ecosystem with special reference to active natural hydrocarbon seepage. *Marine Chemistry*, 118(3-4), pp.140-155.

Burns, K.A. and Jones, R., 2016. Assessment of sediment hydrocarbon contamination from the 2009 Montara oil blow out in the Timor Sea. *Environmental Pollution*, 211, pp.214-225.

ECHA, 2017, Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7c: Endpoint specific guidance, Version 3.0, March 2017. https://echa.europa.eu/documents/10162/23047722/ir_csa_r7c_pbt_caracal_draft_en.pdf/2510b293-f8e4-1f85-13b4-84094632ffa3 date accessed: 29th June 2021.

Elfadly, A.A., Ahmed, O.E. and El Nady, M.M., 2017. Assessing of organic content in surface sediments of Suez Gulf, Egypt depending on normal alkanes, terpanes and steranes biological markers indicators. *Egyptian Journal of Petroleum*, 26(4), pp.969-979.

Folmar, L.C., Craddock, D.R., Blackwell, J.W., Joyce, G. and Hodgins, H.O., 1981. Effects of petroleum exposure on predatory behavior of coho salmon (*Oncorhynchus kisutch*). *Bulletin of environmental contamination and toxicology*, *27*(1), pp.458-462.

Forth, H.P., Mitchelmore, C.L., Morris, J.M. and Lipton, J., 2017. Characterization of oil and water accommodated fractions used to conduct aquatic toxicity testing in support of the Deepwater Horizon oil spill natural resource damage assessment. *Environmental Toxicology and Chemistry*, 36(6), pp.1450-1459.

Fritt-Rasmussen, Janne, Susse Wegeberg, Kim Gustavson, Kristin Rist Sørheim, Per S. Daling, Kirsten Jørgensen, Ossi Tonteri, and Jens Peter Holst-Andersen. *Heavy Fuel Oil (HFO): A review of fate and behaviour of HFO spills in cold seawater, including biodegradation, environmental effects and oil spill response*. Nordic Council of Ministers, 2018.

Gagnon, M.M. and Holdway, D.A., 2002. EROD activity, serum SDH and PAH biliary metabolites in sand flathead (*Platycephalus bassensis*) collected in Port Phillip Bay, Australia. *Marine pollution bulletin*, *44*(3), pp.230-237.

Gagnon, M.M. and Rawson, C., 2012. Montara well release, monitoring study S4A phase IV assessment of effects on Timor Sea fish. Curtin University, Perth, Western Australia.

Gagnon, M.M. and Rawson, C.A., 2017. Bioindicator species for EROD activity measurements: A review with Australian fish as a case study. *Ecological Indicators*, *73*, pp.166-180.

Gissi, A., Lombardo, A., Roncaglioni, A., Gadaleta, D., Mangiatordi, G.F., Nicolotti, O. and Benfenati, E., 2015. Evaluation and comparison of benchmark QSAR models to predict a relevant REACH endpoint: the bioconcentration factor (BCF). *Environmental research*, *137*, pp.398-409.

Gregg, J.C., Fleeger, J.W. and Carman, K.R., 1997. Effects of suspended, diesel-contaminated sediment on feeding rate in the darter goby, *Gobionellus boleosoma* (Teleostei: Gobiidae). *Marine Pollution Bulletin*, *34*(4), pp.269-275.

Grey, D.L., 1987. An overview of *Lates calcarifer* in Australia and Asia. *Management of wild and cultured sea bass/barramundi*, pp.15-21.

González, J.J., Viñas, L., Franco, M.A., Fumega, J., Soriano, J.A., Grueiro, G., Muniategui, S., López-Mahía, P., Prada, D., Bayona, J.M. and Alzaga, R., 2006. Spatial and temporal distribution of dissolved/dispersed aromatic hydrocarbons in seawater in the area affected by the Prestige oil spill. *Marine Pollution Bulletin*, *53*(5-7), pp.250-259.

Di Giulio, R.T. and Hinton, D.E. eds., 2008. The Toxicology of Fishes. CRC Press.

Hardihn, S. and Hill, J.E., 2012. Risk analysis of Barramundi Perch *Lates calcarifer* aquaculture in Florida. *North American Journal of Fisheries Management*, *32*(3), pp.577-585.

Heintz, R.A., Short, J.W. and Rice, S.D., 1999. Sensitivity of fish embryos to weathered crude oil: Part II. Increased mortality of pink salmon (*Oncorhynchus gorbuscha*) embryos incubating downstream from weathered Exxon Valdez crude oil. *Environmental Toxicology and Chemistry*, 18(3), pp.494-503.

Hellou, J., Leonard, J. and Anstey, C., 2002. Dietary exposure of finfish to aromatic contaminants and tissue distribution. *Archives of Environmental Contamination and Toxicology*, *42*(4), pp.470-476.

Hellou, J. and Leonard, J., 2004. Polycyclic aromatic hydrocarbons bioaccumulation and biotransformation products in trout exposed through food pellets. *Polycyclic Aromatic Compounds*, *24*(4-5), pp.697-712.

Hodson, P. V., Kloepper-Sams, P. J., Munkittrick, K. R., Lockhart, W. L., Metner, D. A., Luxon, P. L., Smith, I. R., Gagnon, M. M., Servos, M., Payne, J. F., 1991. Protocols for Measuring Mixed Function Oxygenases of Fish Livers. *Canadian Technical Report of Fisheries and Aquatic Sciences* 1829, 51 p.

Hunter, T., 2010. The Montara Oil Spill and the National Marine Oil Spill Contingency Plan: Disaster Response or Just a Disaster. *Austalian. & New Zealand Maritime Law Journal*, *24*, p.46.

Husson, F., Josse, J., Le, S., Mazet, J. and Husson, M.F., 2016. Package 'FactoMineR'. An R package, 96, p.698.

Kasumyan, A.O., 2001. Effects of chemical pollutants on foraging behavior and sensitivity of fish to food stimuli. *Journal of Ichthyology*, *41*(1), pp.76-87.

Law, R.J. and Hellou, J., 1999. Contamination of fish and shellfish following oil spill incidents. *Environmental Geosciences*, *6*(2), pp.90-98.

Lê, Sébastien, Julie Josse, and François Husson. "FactoMineR: an R package for multivariate analysis." *Journal of Statistical Software* 25, no. 1 (2008): 1-18.

Lewis, A. 2002. Composition, properties and classification of heavy fuel oils. Third R&D Forum on High-density Oil Spill Response, Brest. March 2002 pp.11–25.

Lin, E.L., Cormier, S.M. and Torsella, J.A., 1996. Fish biliary polycyclic aromatic hydrocarbon metabolites estimated by fixed-wavelength fluorescence: comparison with HPLC-fluorescent detection. *Ecotoxicology and Environmental Safety*, *35*(1), pp.16-23.

Lombardo, A., Roncaglioni, A., Boriani, E., Milan, C. and Benfenati, E., 2010, July. Assessment and validation of the CAESAR predictive model for bioconcentration factor (BCF) in fish. In *Chemistry Central Journal* (Vol. 4, No. S1, p. S1). Springer International Publishing.

Mathew, G., 2019. Taxonomy, identification and biology of Seabass (*Lates calcarifer*). Central Marine Fisheries Research Institute. *India 43p*.

Marchand, J., Quiniou, L., Riso, R., Thebaut, M.T. and Laroche, J., 2004. Physiological cost of tolerance to toxicants in the European flounder *Platichthys flesus*, along the French Atlantic Coast. *Aquatic Toxicology*, 70(4), pp.327-343.

Mercurio, P., Burns, K.A. and Cavanagh, J., 2004. Testing the ecotoxicology of vegetable versus mineral based lubricating oils: 2. Induction of mixed function oxidase enzymes in barramundi, Lates calcarifer, a tropical fish species. *Environmental Pollution*, *129*(2), pp.175-182.

McKim, J. M. (1994). Physiological and biochemical mechanisms that regulate the accumulation and toxicity of environmental chemicals in fish. In J. L. Hamelink, P. F. Landrum, H. L. Bergman, and W. H. Benson (Eds.), *Bioavailability: Physical, biological and chemical interactions* (pp. 179–201). Boca Raton, FL: CRC Press.

Morimoto, R.I., 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes & Development*, *12*(24), pp.3788-3796.

Murawski, S.A., Hogarth, W.T., Peebles, E.B. and Barbeiri, L., 2014. Prevalence of external skin lesions and polycyclic aromatic hydrocarbon concentrations in Gulf of Mexico fishes, post-Deepwater Horizon. Transactions of the American Fisheries Society, 143(4), pp.1084-1097.

523 Nahrgang, J., Camus, L., Gonzalez, P., Jönsson, M., Christiansen, J.S. and Hop, H., 2010. Biomarker
524 responses in polar cod (Boreogadus saida) exposed to dietary crude oil. *Aquatic Toxicology*, *96*(1),
525 pp.77-83.

526 Nunes, B.S., Travasso, R., Gonçalves, F. and Castro, B.B., 2015. Biochemical and physiological
527 modifications in tissues of *Sardina pilchardus*: spatial and temporal patterns as a baseline for
528 biomonitoring studies. *Frontiers in Environmental Science*, *3*, p.7.

529 OSPAR Commission, The Convention for the Protection of the Marine Environment of the North-East
530 Atlantic, Background documents and technical annexes for biological effects monitoring, 2013.

531 Prahalad, A.K., Inmon, J., Ghio, A.J. and Gallagher, J.E., 2000. Enhancement of 2 '-Deoxyguanosine
532 Hydroxylation and DNA Damage by Coal and Oil Fly Ash in Relation to Particulate Metal Content and
533 Availability. *Chemical Research in Toxicology*, *13*(10), pp.1011-1019.

534 Rao, J.V., Begum, G., Pallela, R., Usman, P.K. and Rao, R.N., 2005. Changes in behavior and brain
535 acetylcholinesterase activity in mosquito fish, Gambusia affinis in response to the sub-lethal
536 exposure to chlorpyrifos. *International Journal of Environmental Research and Public Health*, *2*(3),
537 pp.478-483.

538 Reynaud, S. and Deschaux, P., 2006. The effects of polycyclic aromatic hydrocarbons on the immune
539 system of fish: a review. *Aquatic toxicology*, *77*(2), pp.229-238.

540 Satheeshkumar, P., Ananthan, G., Kumar, D.S. and Jagadeesan, L., 2012. Haematology and
541 biochemical parameters of different feeding behaviour of teleost fishes from Vellar estuary,
542 India. *Comparative Clinical Pathology*, *21*(6), pp.1187-1191.

543 Scarlett, A.G., Holman, A.I., Georgiev, S.V., Stein, H.J., Summons, R.E. and Grice, K., 2019. Multi-
544 spectroscopic and elemental characterization of southern Australian asphaltites. *Organic*
545 *Geochemistry,* 133, pp.77-91.

Schlenk, D. and Benson, W.H. eds., 2017. Target organ toxicity in marine and freshwater teleosts: Organs. CRC press.

Scott, G.R. and Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. *Aquatic Toxicology*, *68*(4), pp.369-392.

Seveso, D., Louis, Y.D., Montano, S., Galli, P. and Saliu, F., 2021. The Mauritius Oil Spill: What's Next?. *Pollutants*, *1*(1), pp.18-28.

Shigenaka, G. 2014. Twenty-Five Years After the Exxon Valdez Oil Spill: NOAA's Scientific Support, Monitoring, and Research. Seattle: NOAA Office of Response and Restoration. 78 pp

Singer, M.M., Aurand, D., Bragin, G.E., Clark, J.R., Coelho, G.M., Sowby, M.L. and Tjeerdema, R.S., 2000. Standardization of the preparation and quantitation of water-accommodated fractions of petroleum for toxicity testing. *Marine Pollution Bulletin*, *40*(11), pp.1007-1016.

Snyder, R.A., Vestal, A., Welch, C., Barnes, G., Pelot, R., Ederington-Hagy, M. and Hileman, F., 2014. PAH concentrations in Coquina (Donax spp.) on a sandy beach shoreline impacted by a marine oil spill. *Marine Pollution Bulletin*, *83*(1), pp.87-91.

Smeltz, M., Rowland-Faux, L., Ghiran, C., Patterson III, W.F., Garner, S.B., Beers, A., Mièvre, Q., Kane, A.S. and James, M.O., 2017. A multi-year study of hepatic biomarkers in coastal fishes from the Gulf of Mexico after the Deepwater Horizon oil spill. *Marine Environmental Research*, *129*, pp.57-67.

Smith, E.L., Rowland, S.J., Galloway, T. and Scarlett, M.A., 2006. Potential Ecological Effects of Chemically Dispersed and Biodegraded Oils Evaluation of components and concentrations relevant to policy decisions. *Maritime and Coast Guard Agency UK Report*, (562).

Spies, R.B., Mukhtasor, M. and Burns, K.A., 2017. The Montara oil spill: a 2009 well blowout in the Timor Sea. *Archives of Environmental Contamination and Toxicology*, *73*(1), pp.55-62.

569 Stehr, C.M., Myers, M.S., Johnson, L.L., Spencer, S. and Stein, J.E., 2004. Toxicopathic liver lesions in
570 English sole and chemical contaminant exposure in Vancouver Harbour, Canada. *Marine*
571 *Environmental Research*, *57*(1-2), pp.55-74.

572 Torreiro-Melo, A. G. A. G., Silva, J. S., Bianchini, A., Zanardi-Lamardo, E., Carvalho, P. S. M., 2015.
573 Bioconcentration of phenanthrene and metabolites in bile and behavioral alterations in the tropical
574 estuarine guppy *Poecilia vivipara*. *Chemosphere*. 132:17-23.

575 Triebskorn, Rita, Heinz-R. Köhler, Wolfgang Honnen, Michael Schramm, S. Marshall Adams, and
576 Ewald F. Müller. "Induction of heat shock proteins, changes in liver ultrastructure, and alterations of
577 fish behavior: are these biomarkers related and are they useful to reflect the state of pollution in the
578 field?." *Journal of Aquatic Ecosystem Stress and Recovery* 6, no. 1 (1997): 57-73.

579 Turner, R.E., Overton, E.B., Meyer, B.M., Miles, M.S. and Hooper-Bui, L., 2014. Changes in the
580 concentration and relative abundance of alkanes and PAHs from the Deepwater Horizon oiling of
581 coastal marshes. *Marine Pollution Bulletin*, *86*(1-2), pp.291-297.

582 Uhler, A.D., Stout, S.A., Douglas, G.S., Healey, E.M. and Emsbo-Mattingly, S.D., 2016. Chemical
583 character of marine heavy fuel oils and lubricants. In *Standard Handbook Oil Spill Environmental*
584 *Forensics* (pp. 641-683). Academic Press.

585 Valavanidis, A., Vlachogianni, T. and Fiotakis, C., 2009. 8-hydroxy-2'-deoxyguanosine (8-OHdG): a
586 critical biomarker of oxidative stress and carcinogenesis. *Journal of Environmental Science and*
587 *Health Part C*, *27*(2), pp.120-139.

588 Van der Oost, R., Beyer, J. and Vermeulen, N.P., 2003. Fish bioaccumulation and biomarkers in
589 environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, *13*(2), pp.57-
590 149.

591 Varanasi, U., 1989. *Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment*. CRC
592 press.

593 Veith, G.D., DeFoe, D.L. and Bergstedt, B.V., 1979. Measuring and estimating the bioconcentration
594 factor of chemicals in fish. *Journal of the Fisheries Board of Canada*, 36(9), pp.1040-1048.

595 Vieweg, I., Bilbao, E., Meador, J.P., Cancio, I., Bender, M.L., Cajaraville, M.P. and Nahrgang, J., 2018.
596 Effects of dietary crude oil exposure on molecular and physiological parameters related to lipid
597 homeostasis in polar cod (*Boreogadus saida*). *Comparative Biochemistry and Physiology Part C:*
598 *Toxicology & Pharmacology*, *206*, pp.54-64.

599 Vignet, C., Le Menach, K., Mazurais, D., Lucas, J., Perrichon, P., Le Bihanic, F., Devier, M.H., Lyphout,
600 L., Frère, L., Bégout, M.L. and Zambonino-Infante, J.L., 2014 (a). Chronic dietary exposure to pyrolytic
601 and petrogenic mixtures of PAHs causes physiological disruption in zebrafish-part I: Survival and
602 growth. *Environmental Science and Pollution Research*, *21*(24), pp.13804-13817.

603 Vignet, C., Le Menach, K., Lyphout, L., Guionnet, T., Frère, L., Leguay, D., Budzinski, H., Cousin, X. and
604 Bégout, M.L., 2014 (b). Chronic dietary exposure to pyrolytic and petrogenic mixtures of PAHs causes
605 physiological disruption in zebrafish—part II: behavior. *Environmental Science and Pollution*
606 *Research*, *21*(24), pp.13818-13832.

607 Voutsas, E., Magoulas, K. and Tassios, D., 2002. Prediction of the bioaccumulation of persistent
608 organic pollutants in aquatic food webs. *Chemosphere*, *48*(7), pp.645-651.

609 Wang, Z., Liu, Z., Xu, K., Mayer, L.M., Zhang, Z., Kolker, A.S. and Wu, W., 2014. Concentrations and
610 sources of polycyclic aromatic hydrocarbons in surface coastal sediments of the northern Gulf of
611 Mexico. *Geochemical Transactions*, *15*(1), pp.1-12.

612 Weis, J.S. and Candelmo, A., 2012. Pollutants and fish predator/prey behavior: a review of laboratory
613 and field approaches. *Current Zoology*, *58*(1), pp.9-20.

614 Whitehead, A., 2013. Interactions between oil-spill pollutants and natural stressors can compound
615 ecotoxicological effects. *Integrative and Comparative Biology*, 53(4), pp.635-647.

616 Woltering, M., Tulipani, S., Boreham, C.J., Walshe, J., Schwark, L. and Grice, K., 2016. Simultaneous
617 quantitative analysis of Ni, VO, Cu, Zn and Mn geoporphyrins by liquid chromatography-high
618 resolution multistage mass spectrometry: Method development and validation. *Chemical Geology*,
619 441, pp.81-91.

620 Wood, C.M., 2011. An introduction to metals in fish physiology and toxicology: basic principles.
621 In *Fish Physiology* (Vol. 31, pp. 1-51). Academic Press.

622 Whyte, J.J., Jung, R.E., Schmitt, C.J. and Tillitt, D.E., 2000. Ethoxyresorufin-O-deethylase (EROD)
623 activity in fish as a biomarker of chemical exposure. *Critical Reviews in Toxicology*, *30*(4), pp.347-570.

624 Yue, G.H., Zhu, Z.Y., Lo, L.C., Wang, C.M., Lin, G., Feng, F., Pang, H.Y., Li, J., Gong, P., Liu, H.M. and
625 Tan, J., 2009. Genetic variation and population structure of Asian seabass (*Lates calcarifer*) in the
626 Asia-Pacific region. *Aquaculture*, *293*(1-2), pp.22-28.

627 Table 1: Selected Metals and Total PAHs measured in MCO and HFO.

	Compound	MCO (mg/kg)	HFO (mg/kg)
Total PAHs*	Naphthalenes (C1-C4)	29800 ± 1180	11900 ± 124
	Phenanthrenes (C1-C4)	6370 ± 210	4830 ± 39
	Pyrenes/Fluoranthenes	910 ± 22	2550 ± 49
	Benzopyrenes/Benzofluoranthenes	0 ± 0	891 ± 29
	Dibenzothiophenes	1270 ± 46	3530 ± 69
	Chrysenes	61 ± 2	2970 ± 29
Metals	Aluminium	30.70 ± 17.7	15.44 ± 8.91
	Arsenic	< 0.03	0.04 ± 0.02
	Barium	0.11 ± 0.06	1.32 ± 0.76
	Chromium	0.89 ± 0.52	0.24 ± 0.14
	Cobalt	< 0.46	2.15 ± 1.24
	Copper	0.45 ± 0.26	< 0.31
	Iron	4.73 ± 2.73	37.90 ± 21.8
	Lead	0.08 ± 0.05	0.04 ± 0.02
	Molybdenum	< 0.01	0.05 ± 0.03
	Nickel	0.11 ± 0.06	12.23 ± 7.06
	Silver	< 0.01	< 0.01
	Sulfur	394 ± 227	10200 ± 5900
	Tin	0.18 ± 0.1	0.13 ± 0.07
	Titanium	< 0.24	3.24 ± 1.87
	Vanadium	< 0.03	15.27 ± 8.81
	Zinc	1.47 ± 0.85	1.19 ± 0.69

628 *Total PAH is defined as the sum of parent compounds plus all alkylated C1, C2, C3 and C4
629 homologues.
630 Values denoted with "<" were below the stated limit of reporting (see Tables S1, S2 and S3).
631 A full list of all PAHs and metals included in the analytical suites, and the analysis of fish food spiked
632 with oil, is provided in the supplementary information.

633

634 Table 2: Baseline values of 11 Biomarkers for healthy juvenile *Lates calcarifer.*

Biomarker	***Lates calcarifer* Baseline Range**
Condition Factor	15.58 - 16.61
Hepatosomatic Index	1.45 - 1.90
Haematocrit	0.25 - 0.33
Naphthalene-type Biliary Metabolites (μg/mg protein)	3.30 - 4.18
Phenanthrene-type Biliary Metabolites (μg/mg protein)	20.69 - 26.55
Pyrene-type Biliary Metabolites (μg/mg protein)	1.45 - 1.78
Benzo(*a*)pyrene-type Biliary Metabolites (μg/mg protein)	6.91 - 8.48
DNA Damage (as 8-oxo-dG) ng/mg protein)	0.78 - 0.97
AChE (ng/mg protein)	0.82 - 0.98
HSP70 (pg/mg protein)	2.95 - 4.51
EROD (pmol/min/mg protein)	0.80 - 1.11

635

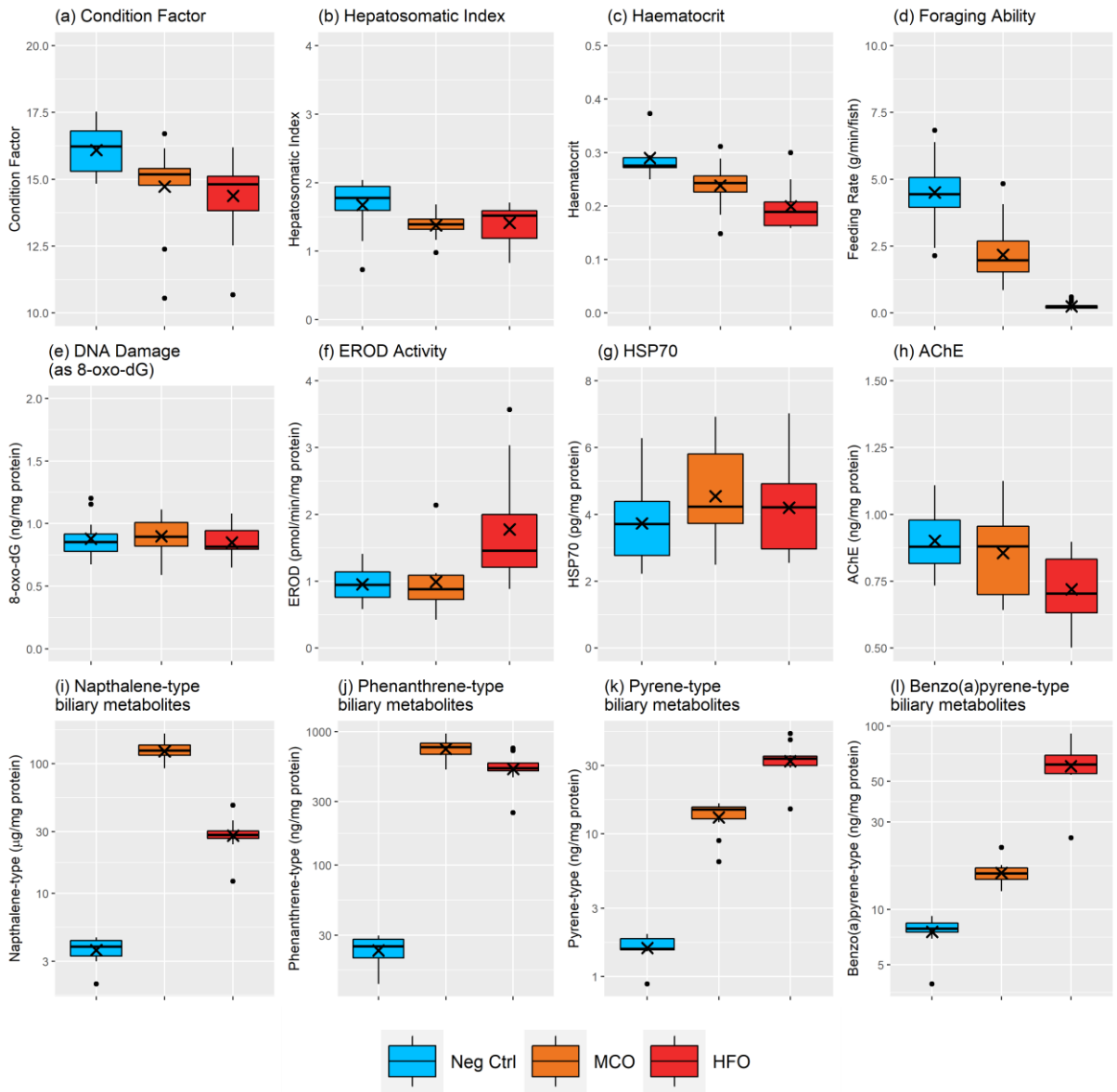
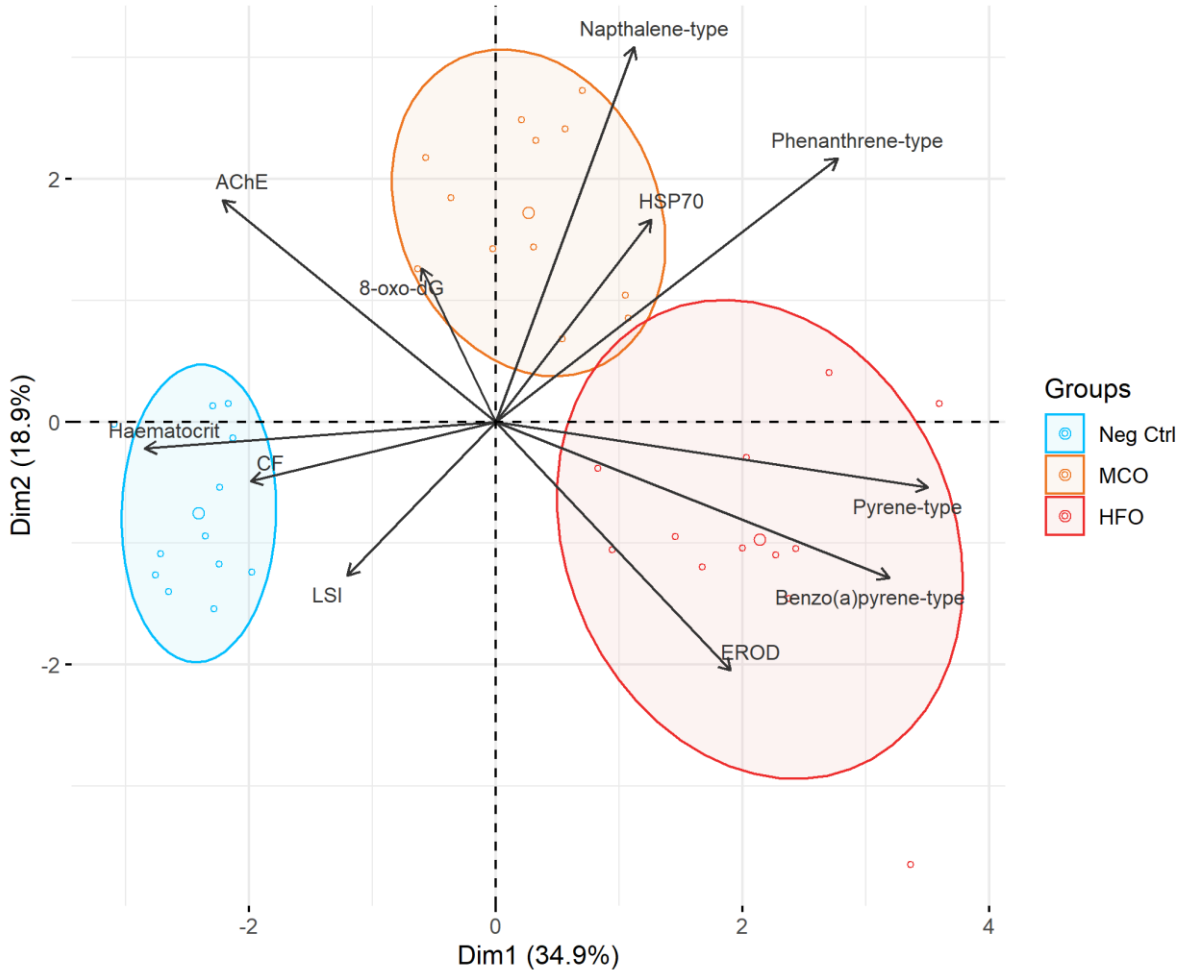


Figure 1: Boxplots of 12 biomarker responses of *Lates calcarifer* exposed to petroleum hydrocarbons.

Lines are the median, the means are denoted by 'x', and dots are outliers.

* indicates result statistically significantly different from negative control ($p \leq 0.05$).



641

642 Figure 2: PCA biplot of biomarker profiles of L. calcarifer exposed to petroleum hydrocarbons.

643