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1 **The effects of hypoxia on aerobic metabolism in oil-contaminated**
2 **sea bass (*Dicentrarchus labrax*)**

3
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6
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16
17 **Abstract**

18 Hypoxia and petrogenic hydrocarbon contamination are two anthropogenic stressors that
19 coexist in coastal environments. Although studies have estimated the impact of each stressor
20 separately, few investigations have assessed the effects of these stressors in interaction. We
21 therefore investigated the impact of these combined stressors on sea bass, (*Dicentrarchus*
22 *labrax*) physiology. After experimental contamination with physically dispersed oil, fish were
23 exposed to hypoxia or normoxia, and active/standard metabolic rates (AMR and SMR,
24 respectively), and metabolic scope (MS) were estimated. At the protocol's end, the uptake of

25 polycyclic aromatic hydrocarbons (PAHs) was estimated by evaluating relative
26 concentrations of bile metabolites. In terms of bile metabolites, our results validated the
27 uptake of PAHs by contaminated fish in our experimental settings, and further suggest that
28 the hypoxic period after contamination does not reduce or increase compound metabolization
29 processes. Our data showed significant effects of hypoxia on all metabolic rates: a significant
30 drastic AMR reduction and significant SMR diminution led to decreased MS. We also found
31 that oil contamination significantly impacted AMR and MS, but not SMR. These results
32 suggested that when evaluated separately, hypoxia or oil affect the metabolic rate of sea bass.
33 On the other hand, when evaluated in combination, no cumulative effects were observed,
34 since fish exposed to both stressors did not show a stronger impact on metabolism than fish
35 exposed to hypoxia alone. This suggests that oil impacts fish metabolism when fish occupy
36 normoxic waters, and that oil does not magnify hypoxia-induced effects on fish metabolism.

37

38 **Keywords:** oil, hypoxia, *Dicentrarchus labrax*, metabolic scope, biliary metabolites, active
39 metabolic rate

40

41 **1. Introduction**

42 While coastal ecosystems contribute to the economic value and biodiversity of marine
43 environments (Costanza *et al.*, 1997; Worm *et al.*, 2006), these habitats are under multiple
44 anthropogenic stressors. Hypoxia is one such threatening stressor, as hypoxic areas have
45 exponentially increased, globally, since the 1960s (Breitburg *et al.*, 2018). This is mainly due
46 to growing population centres that generate excessive nutrient outputs, leading to the
47 eutrophication of local ecosystems (Diaz and Rosenberg, 2008). Nowadays, more than 400
48 hypoxic ecosystems are recognised and distributed world-wide (Selman *et al.*, 2008). In
49 general, major hypoxic events (i.e. dissolved oxygen levels < 0.5 mL/L, Diaz and Rosenberg,

50 1995) are publicised, as a mass mortality of aquatic organisms often occurs at these locations
51 (e.g. Nakano *et al.*, 2017; Altieri *et al.*, 2017). However, sub-lethal hypoxic effects, induced
52 by less intense events, are much more frequent (Diaz and Rosenberg, 1995). In this
53 framework, multiple studies have reported on the impact of hypoxia on several fitness
54 proxies, including fish metabolism (e.g. Claireaux *et al.*, 2000; Claireaux and Chabot, 2016;
55 Rosewarne *et al.*, 2016), locomotor performance (e.g. Lefrançois *et al.*, 2005; Lefrançois and
56 Domenici, 2006; Domenici *et al.*, 2007) and fish growth (e.g. Pichavant *et al.*, 2000; McNatt
57 and Rice, 2004; Jeppesen *et al.*, 2018).

58
59 Hypoxia is not the only stressor affecting marine communities. Petrogenic hydrocarbon
60 contamination is prevalent on a worldwide scale, and is derived from different sources such as
61 shipwrecks, oil-spills, operational discharges and offshore exploration (GESAMP, 2009).
62 These issues are relevant since new oil extraction techniques (“unconventional techniques”)
63 increase the risk of oil released at sea (GESAMP, 2015; Harriss, 2016; Shearer, 2018). The
64 effects of petrogenic hydrocarbons have been extensively investigated at several levels of
65 biological organisation, in different taxa (Bejarano and Michel, 2016). However, marine
66 ecosystems are rarely under pressure from single factors, and biological impacts are more
67 often the result of synergistic environmental and anthropogenic stressors.

68
69 The northern Gulf of Mexico is a well-known example of hypoxia and petrogenic
70 hydrocarbon coexistence. Indeed, while organisms are commonly exposed to hypoxia, thanks
71 to nutrient discharge from the Mississippi and Atchafalaya Rivers (Diaz and Rosenberg,
72 2008), they also face petroleum contamination due to oil spills, such as Deep Water Horizon
73 (White *et al.*, 2012) and operational discharges (Wang *et al.*, 2014). In terms of the scientific
74 literature on this subject, evaluating the combined effects of hypoxia and oil is still a new area

75 of research, where more investigation is required. Nonetheless, limited studies are available
76 and have investigated the physiological effects of both stressors in teleost fish (Negreiros *et*
77 *al.*, 2011; Claireaux *et al.*, 2013; Dasgupta *et al.*, 2015; Mager *et al.*, 2018; Milinkovitch *et*
78 *al.*, 2019). Among them, some of these studies have challenged organisms with severe levels
79 of hypoxia and/or contamination, sporadically encountered on the field (Claireaux *et al.*,
80 2013; Dasgupta *et al.*, 2015). Others assessed biological effects at sub-individual levels (e.g.
81 Negreiros *et al.*, 2011), consequently, impact predictions on organism fitness are uncertain.

82

83 Therefore, we sought to experimentally evaluate the effects of hypoxia on the metabolic scope
84 (MS) of the European Sea bass (*Dicentrarchus labrax*), previously contaminated with oil. MS
85 was defined as the difference between the active metabolic rate (AMR) and the standard
86 metabolic rate (SMR), and was chosen as a biological parameter of interest, since it estimates
87 the instantaneous energy an organisms allocates to demanding activities (i.e. locomotion,
88 digestion, feeding, etc.), and is therefore deemed an optimal, measurable fitness proxy
89 (Claireaux and Lefrançois, 2007). Moreover, MS is modulated by hypoxic environments
90 (Farrell and Richards, 2009) and oil exposure (Mager *et al.*, 2014), but no studies have
91 investigated the cumulative effects of both stressors on sea bass metabolism. Sea bass
92 (*Dicentrarchus labrax*) was chosen as a biological model thanks to several key
93 characteristics; its primary role as a marine predator, its economic and commercial relevance
94 (aquaculture production of 165, 915 tons and capture fisheries of 6,919 tons in 2016; FAO,
95 2018), and its perceived sensitivity to hypoxia (Terova, 2008) and hydrocarbons (Claireaux *et*
96 *al.*, 2018).

97

98 **2. Materials and Methods**

99

100 **2.1. Animals**

101 Forty-four juvenile sea bass, aged 1.5–2 years old were used. Fish were provided by
102 Aquanord Ichtus (Gravelines, France). Sea bass were acclimated in 2 m³ tanks, with an open
103 circulation of sea water under natural photoperiods of six weeks. Dissolved oxygen
104 concentrations, salinity, temperature and pH were monitored daily [$93.23 \pm 0.77\%$ air
105 saturation, $35.30 \pm 0.06\%$, $21.28 \pm 0.09^{\circ}\text{C}$ and 7.96 ± 0.02 , respectively (mean \pm standard
106 error (S.E)]. Fish were fed with Neostart (Le Gouessant, France), and food was withheld 24 h
107 before exposure to contaminants (see below). The average fish length and weight were, 15.06
108 ± 0.13 cm and 35.27 ± 0.88 g, respectively.

109

110 **2.2. Exposure to oil and total petroleum hydrocarbons (TPHs) measurement**

111 A Brazilian crude oil was used in this study; the PAH concentration was previously described
112 (Milinkovitch *et al.*, 2019). Briefly, the concentration of 21 US-EPA PAHs (parent and
113 alkylated) was 8.264 mg PAHs per g petroleum. The viscosity at 20°C was 35,574 mPa and
114 the pour and flash points were -28°C and -2.5°C, respectively.

115

116 The contamination set-up was described by previous studies (Milinkovitch *et al.*, 2011a, b;
117 Claireaux *et al.*, 2013; Milinkovitch *et al.*, 2015; Dussauze *et al.*, 2015 ; Geraudie *et al.*, 2016;
118 Milinkovitch *et al.*, 2019). Briefly, a water pump linked to a funnel, continuously sucked up
119 petroleum and/or seawater from the surface and expelled the mixture to the bottom of a 300 L
120 tank. This generated a homogenous dispersion of oil in the water column, after a period of 6 h
121 (Dussauze *et al.*, 2015).

122

123 In this study, 15 g oil (or 15 g seawater for the control treatment) were poured into the funnel
124 and after 6 h, pairs of fish were exposed for 18 h. Every day, two fish were exposed to oil

125 treatments, and two fish were exposed to the control treatment (four fish exposed per day). In
126 total, we carried out eleven days of exposure; 22 fish were exposed to oil treatments, and 22
127 fish were exposed to control treatments. During the exposure period, temperature, salinity, pH
128 and dissolved oxygen were stable: $21.30^{\circ}\text{C} \pm 0.10$, $35.06 \pm 0.10\text{‰}$, 8.06 ± 0.01 , 99.60 ± 0.40
129 % air saturation, respectively.

130

131 For each exposure, three 125 mL samples were collected from the tank bottom at the
132 beginning (T = 0 h) and at the end of exposure (T = 18 h) to measure TPH concentration.
133 Chemical assays were conducted according to Fusey and Oudot (1976). Briefly, samples were
134 extracted using 25 mL dichloromethane (Carlo Erba Reactifs, France), thus enabling
135 separation, by decantation of organic and aqueous phases. The organic phase was removed
136 and dried on anhydrous sulphate to avoid residual from the aqueous phase. Then, extracts
137 were analysed at 390 nm, on a UV spectrophotometer (Unicam, France). The limit of
138 quantification was $< 1 \text{ mg/L}$.

139

140 **2.3. Respirometry measurements under hypoxic or normoxic conditions**

141 Every day, of the four fish exposed to oil or control treatments, two were individually
142 exposed to hypoxia and two were individually exposed to normoxia. Finally, four groups of
143 11 fish were generated: 1) fish previously exposed to control treatment, and then normoxic
144 conditions (Ctrl norx group); 2) fish previously exposed to control treatment, and then
145 hypoxic conditions (Ctrl hyp group); 3) fish previously exposed to oil treatment, and then
146 normoxic conditions (Oil norx group) and 4) fish previously exposed to oil treatment, and
147 then hypoxic conditions (Oil hyp group). Practically, in order to expose fish to normoxia or
148 hypoxia, each contaminated fish was immediately transferred with a net (air exposure $< 10 \text{ s}$)
149 to a 0.5 L cylindrical static respirometry chamber (Loligo Systems, Denmark), and immersed

150 in a 100 L clean sea water tank ($21.50 \pm 0.06^\circ\text{C}$). Before any respirometry measurements,
151 normoxic or hypoxic conditions were established. Normoxic conditions were generated by a
152 constant bubbling of air into the tank. This allowed the oxygen concentration to persist at
153 percentages $> 95\%$ air saturation ($6.84 \text{ mg O}_2/\text{L}$). In contrast, hypoxic conditions were
154 generated by bubbling nitrogen into the tank, and monitoring levels through an oxygen probe
155 that transferred this information to an oxygen regulator (OXY-REG, Loligo Systems,
156 Denmark), linked to a solenoid valve (Gas control set, Loligo Systems, Denmark). Depending
157 on tank oxygen levels, the regulator allowed the bubbling of nitrogen (*i.e.* the solenoid valve
158 was open) from a nitrogen gas bottle in a gas equilibration column provided with seawater
159 from the tank (tanks to a water pump EHEIM 600, UK). Deoxygenated water was then sent
160 back into the tank. For these hypoxic conditions, the oxygen regulator was set at 25% air
161 saturation ($1.80 \text{ mg O}_2/\text{L}$), resulting in homogenous concentrations between 24–26% air
162 saturation ($1.72 \text{ mg O}_2/\text{L}$ and $1.87 \text{ mg O}_2/\text{L}$, respectively). Oxygen reduction was performed
163 during 3 h to generate a progressive oxygen decline (Lefrançois and Domenici, 2006), to
164 allow fish to acclimate to hypoxic conditions.

165 After 3 h (performed for fish under normoxic and hypoxic conditions), metabolic rates were
166 measured by intermittent flow respirometry: an external pump that initially allowed for the
167 transfer of water from the tank into the respirometer was turned off every 30 min, for a 15
168 min duration. The respirometer was in a closed state, and oxygen consumption was measured
169 every 15 s with a dipping probe mini sensor (Loligo Systems, Denmark), associated with
170 optodes (Oxy-4 mini; PreSens Precision Sensing GmbH, Regensburg, Germany) and
171 appropriate software (Pre-Sens Oxy 4v2). Oxygen consumption was calculated using linear
172 least-squares regression (excluding the first and last 2 min of each 15 min closed phase) and
173 expressed as $\text{mg of O}_2 \cdot \text{h}^{-1} \cdot \text{kg}^{-1}$. Then, to integrate the log-log allometric relationship between

174 SMR and body mass, data were corrected to a fish body weight of 100 g, using 0.77 as a
175 weight exponent (Claireaux *et al.*, 2006).

176

177 Fish were left in the respirometer for 24 h, and oxygen consumption was measured every 30
178 min. SMR was evaluated after 20 h, using the 15th percentile method (Chabot and Claireaux,
179 2008). AMR was measured after 24 h – with only 4 h between SMR and AMR measurements,
180 thereby preventing a decrease of PAHs body burden (see **4.1.** for more details)-. In order to
181 measure the AMR, fish were removed from the respirometer and placed in a 10 L bucket,
182 where they were chased to exhaustion. Fish were considered exhausted when they did not
183 respond to gentle tail pinching- and then subjected to brief air exposure (30 s) (Roche *et al.*,
184 2013). They were immediately placed in the static respirometer, where oxygen consumption
185 was measured using linear least-squares regression, and data corrected by fish body mass
186 using the log-log allometric relationship between oxygen consumption and body mass.
187 Finally, MS was calculated for each fish as the difference between AMR and SMR. After
188 AMR measurements, fish were euthanised with eugenol (4-allyl-2-methoxyphenol, Sigma
189 Aldrich, France) at 0.2 mL/L in sea water. The gallbladder was then excised and frozen at -
190 80°C. Bile from the gallbladder was subsequently used to measure PAH metabolites.

191

192 **2.4. Fixed wavelength fluorescence analysis of bile to estimate PAH metabolites**

193 Bile was removed from the gallbladder and diluted in absolute ethanol (Sigma Aldrich) at a
194 ratio of 1:500. Assays were conducted on a spectrofluorimeter (FS5, Edinburgh Instruments,
195 UK), for five fixed wavelength fluorescence (FF) analysis of bile. Naphthalene, pyrene,
196 benzo[a] pyrene-derived metabolites were detected at emission/excitation wavelength spectra
197 of 290 nm/335 nm, 341 nm/383 nm and 380 nm/430 nm, respectively (Aas *et al.*, 2000).
198 Fluorene type derived metabolites were detected at 275 nm/328 nm, according to Kopecka-

199 Pilarczyk and Correia (2009). Phenanthrene type derived metabolites were detected at 250
200 nm/370 nm according to Szlinder-Richert *et al.* (2014). Values were expressed as
201 fluorescence arbitrary units (au).

202

203 **2.5. Statistical analysis**

204 A two-way analysis of variance (ANOVA) was employed to test the effect of
205 “contamination” and “oxygen levels” on SMR, AMR, MS and FF of bile. STATISTICA
206 (version 10, STATSOFT USA) software was used for all analysis, and significance was
207 accepted at $P < 0.05$. If significant effects (of a single factor, of both factors and/or factor
208 interactions) were highlighted, a Fisher *post-hoc* test was performed for intergroup
209 comparisons. Student t-tests highlighted statistical differences between TPHs concentrations
210 at $T = 0$ h and at $T = 18$ h exposure. Results are expressed as mean \pm standard error (S.E).

211

212 **3. Results**

213 **3.1. Hydrocarbon exposure**

214 TPH mean values were 13.6 ± 0.5 mg/L at $T = 0$ h and 14.8 ± 0.8 mg/L at $T = 18$ h. We
215 observed no differences in TPH concentrations for the 11 rounds of fish exposure ($P > 0.05$).
216 We also observed no differences between mean TPH concentrations at 0 h and 18 h ($P >$
217 0.05). Additionally, no TPHs were detected in the water column of the control treatment.

218

219 ANOVA results showed that oil significantly affected the relative concentrations of all PAH
220 types (i.e. naphthalene, fluorene, phenanthrene, pyrene, benzo[a]pyrene) ($P < 0.01$; Figure 1).
221 *Post hoc* tests demonstrated significantly ($P < 0.01$) higher concentrations of metabolites in
222 contaminated fish (Oil norx and Oil hyp), when compared to uncontaminated fish (Ctrl norx
223 and Ctrl hyp). Results showed that hypoxia did not affect the relative concentrations of PAH

224 metabolite types since no significance was highlighted between Oil norx and Oil hyp groups
225 nor between Ctrl norx and Ctrl hyp groups.

226

227 **3.2. Biological effects on metabolic parameters**

228 Our data showed significant effects of oxygen parameters on fish SMR, with mean values for
229 the Ctrl hyp and Oil hyp groups significantly ($P < 0.01$) lower than the Ctrl norx and Oil norx
230 groups (according to *post-hoc* tests). No effects of contamination, nor interactions of factors
231 on SMR were detected ($P > 0.05$).

232 The highest AMR was observed for the Ctrl norx group. AMR for the Oil norx group was
233 significantly lower than the Ctrl norx group ($P < 0.05$). Ctrl hyp and Oil hyp groups generated
234 the lowest AMR: both were significantly lower than the Ctrl norx group ($P < 0.01$ for both
235 comparisons), and both were significantly lower than the Oil norx group ($P < 0.01$ for both
236 comparisons).

237

238 We observed the same patterns for MS; the Ctrl norx group displayed the highest MS; MS
239 was significantly lower for the Oil norx group ($P < 0.05$, when compared to Ctrl norx group);
240 the Ctrl hyp and Oil hyp groups exposed the significantly lowest MS (with $P < 0.01$ i.e. when
241 each group was compared to Ctrl norx or Oil norx groups).

242

243 **4. Discussion**

244 **4.1. TPHs concentrations and PAHs metabolites**

245 Our results revealed TPH concentration stability in the tank of exposure during the 18 h
246 exposure. In addition, TPH concentrations did not show any statistical differences between
247 replicates, demonstrating repeatability of the contamination protocol. Our results also showed
248 that fish exposed to oil treatment were contaminated with 13.6 ± 0.5 mg/L and 14.8 ± 0.8

249 mg/L oil, at T = 0 h and T = 18 h, respectively. This order of magnitude agreed with data
250 from Lewis and Daling (2001) when modelling oil slick dispersion, and with measurements
251 conducted in the field, during oil spills (Lunel, 1995). Moreover, it is interesting to note that
252 ships of 400 gross tonnage and above are allowed to discharge oil contaminated water into the
253 sea if concentration does not exceed 15 mg/L of seawater (Convention Marpol, 1973). Thus,
254 the oil concentrations used in this study were appropriate for investigating the impact of oil
255 spills and operational discharge.

256 In order to investigate the combined effects of oil and hypoxia, we first exposed fish to oil and
257 then to hypoxia for 24h. Although a decrease of hydrocarbon body burden could operate
258 during these 24h, we believe it was not substantial since Dussauze *et al.* (2015) showed that
259 14 days were required to observe an approximate 50% decrease in total PAHs in the muscle
260 of *Dicentrarchus labrax*. Be that as it may, we measured relative biliary metabolite
261 concentrations (at the end of the hypoxic/normoxic period) in order to ensure the differential
262 PAH contamination between contaminated and uncontaminated organisms.

263 Our results showed that all types of metabolites were significantly higher in the bile from fish
264 exposed to oil treatment, when compared to control fish. This validates the uptake by
265 organisms of all types of PAHs studied (i.e. naphthalene, fluorene, phenanthrene, pyrene,
266 benzo[a]pyrene). Bile metabolite incorporation of light PAHs was expected, since these
267 compounds are highly soluble in seawater, and are likely to be incorporated into aquatic
268 organisms by diffusion (Neff, 2002). In contrast, the observed incorporation of heavier and
269 the more hydrophobic PAHs, such as benzo[a]pyrene, did not occur by similar processes,
270 since these compounds have a low solubility in seawater (Neff, 2002). Most likely,
271 incorporation was due to contact between suspended oil droplets and the gill surface
272 (Ramachandran *et al.*, 2006), but we cannot exclude ingestion as a potential mode of
273 incorporation. Benzo[a]pyrene uptake was highly relevant since this PAH has been shown to

274 induce developmental, reproductive and immune toxicity, and is proposed as the most potent
275 carcinogenic PAH molecule (Fox *et al.*, 2013). Our results showed that fish exposed to
276 hypoxia or normoxia, after oil contamination, had the same levels of PAH metabolites in their
277 bile, suggesting that the hypoxic period after contamination did not reduce or increase
278 metabolization processes, and would therefore not modulate hydrocarbon body burden in fish.

279

280 **4.2. The effects of oil contamination on fish MS under hypoxic conditions**

281 Our physiological data were in line with other studies. For example, Claireaux *et al.* (2006)
282 measured AMR and SMR values of 408 and 177 mg of O₂. h⁻¹.kg⁻¹, respectively, for juvenile
283 sea bass acclimated to 22°C in control conditions. Although these values were almost half the
284 values observed in our study, they were in the same order of magnitude. This difference could
285 be attributable to different sizes of fish tested and/or to our methodology, since we determined
286 AMR and SMR by static respirometry, whereas Claireaux *et al.* (2006) used a swimming
287 tunnel.

288

289 Our results initially showed an AMR decrease under hypoxic conditions, which was in
290 agreement with previous studies for teleost fish (Claireaux and Lagardère, 1999; Lefrançois
291 and Claireaux, 2003; Jourdan-Pineau *et al.*, 2009). Indeed, while some compensatory
292 mechanisms exist (e.g. hyperventilation), fish cannot maintain optimal oxygen extraction
293 from seawater, when facing hypoxic conditions (Farrell, 2011). As a consequence, AMR is
294 reduced and other aerobic performances, such as locomotion, are influenced by such
295 reductions (Farrell, 2011). Several studies have observed that moderate hypoxia induces
296 AMR reductions in teleost fish: e.g., Lefrançois *et al.* (2003) and Fitzgibbon *et al.* (2007)
297 showed significant AMR reductions when teleost fish (*Solea solea* and *Argyrosomus*
298 *japonicus*) were exposed to 75% air saturation. In our study, we exposed fish to 25% air

299 saturation (1.80 mg O₂/L at 21.5°C), which induced a substantial significant reduction (67%)
300 in AMR, thereby contributing to a drastic 94% reduction in MS. In accordance with Fry (Fry,
301 1947), such a strong reduction in MS prevents fish from conducting discretionary activities
302 (reproduction, locomotion activities, growth, etc.).

303

304 Additionally, a significant reduction in SMR (26%) under hypoxia, suggests that fish were
305 clearly below their critical oxygen concentration (CrC), at 1.80 mg O₂/ L. Based on a study by
306 Claireaux and Lagardère (1999) in sea bass, we expected our fish to reach their CrC at 1.80
307 mg O₂/L, but not such a decrease below normoxic SMR control values. Based on this
308 reduction of the SMR we can estimate that, this level of hypoxia (25% a.s.), in addition to
309 provoke a drastic reduction in energy for discretionary activities, would also impact on
310 metabolic energy required to maintain mandatory activities, such as respiration and
311 osmoregulation. Thus, these observations suggest that, at this level of hypoxia, survival could
312 be compromised. Based on the literature, it is difficult to estimate if our experimental
313 conditions could impact fish survival on a long or short-term basis. Claireaux *et al.* (2013)
314 exposed *D. labrax* to a drastic decrease in oxygen concentrations (< 20% a.s. after 1 h, and <
315 5% a.s. after 10 h). Using this approach, all fish were near death (loss of equilibrium) after 12
316 h. In contrast, Pichavant *et al.* (2001) observed no death when *D. labrax* was exposed to 3.2
317 mg O₂/L (42.9 % a.s.) over 42 days.

318 In relation to the impact of physically dispersed oil (normoxic conditions), our results
319 revealed that this contamination induced a 20% decrease in AMR, and a consequent 32%
320 decrease in MS. This data suggested a reduction in the metabolic energy required for
321 discretionary activities. These results are in contrast to a previous study (Milinkovitch *et al.*,
322 2012), that showed no effects of physically dispersed oil on AMR in golden grey mullet (*Liza*
323 *aurata*). This discrepancy could be due to different AMR methodologies (static respirometry

324 vs. swimming respirometry) and/or to species specific differences, suggesting that *Liza aurata*
325 may be more resistant to hydrocarbons than *D. labrax*. Different oil (experimental
326 contaminant) origins (Brazilian vs. Arabian extraction), and the resulting different physico-
327 chemical properties could explain these discrepancies. For instance, different oil viscosities
328 may have impacted oil dispersion characteristics, affecting processes such as oil
329 bioaccumulation or the adherence of oil droplets to gill structures, etc. Although we did not
330 explore the cause of these AMR reductions, we hypothesize that there was a negative impact
331 of oil contamination on the gills, as the first target organ. Indeed, several studies have shown
332 histopathological and functional changes in fish gills exposed to oil (Hesni *et al.*, 2011;
333 Agamy, 2013 and Gagnon *et al.*, 1999, Cohen *et al.*, 2001). Since SMR was not impacted by
334 oil, we believe that functional changes may have limited oxygen diffusion when the animal is
335 challenged, i.e. during exercise, but were unimportant enough to alter oxygen diffusion when
336 the animal was resting.

337

338 In terms of the impact of contaminated fish under hypoxia (i.e. the interaction of both
339 stressors), our data revealed a significant 67% reduction in AMR, a drastic 95% reduction in
340 MS, and a significant 24% reduction in SMR, when compared to uncontaminated fish under
341 normoxia (i.e. the Ctrl norx group). These observations suggest a reduction in the fish's
342 energy potential to conduct discretionary, as well as mandatory activities. Surprisingly, the
343 impact on these three metabolic parameters was similar, whether fish under hypoxia were
344 contaminated or not, suggesting that at this level of hypoxia, hydrocarbons had no significant
345 effects on fish metabolism, or that the effects of hydrocarbons were masked by this level of
346 hypoxia. This could be due to the fact that when fish respiration is intense (i.e. during AMR in
347 normoxia), hydrocarbon contamination limits this process by limiting oxygen transfer via the
348 gills, whereas when fish respiration is drastically reduced by hypoxia, hydrocarbon

349 contamination may not be a limiting factor. On this basis, it would be interesting to test the
350 effects of hydrocarbons at moderate degrees of hypoxia (i.e. when respiration is not
351 intensively reduced, and hydrocarbons are a limiting factor) to highlight the synergistic or
352 cumulative effects of both stressors in these environments. Mager *et al.* (2018) showed a
353 similar effect of hypoxia alone or combined with oil, on the critical swimming speed of Mahi-
354 mahi, but no effects when fish were exposed to hydrocarbons alone. The degree of
355 hydrocarbon contamination used by Mager *et al.* (2018) was lower than our study, which
356 could explain the lack of effect of this contaminant.

357

358 In conclusion, our study showed an effect of hypoxia on all metabolic parameters, including
359 MS, suggesting that fitness is impacted at oxygen values ≤ 1.8 mg/L. Additionally, AMR and
360 consequently MS were also decreased due to oil contamination under normoxia. Surprisingly,
361 no synergistic or cumulative effects of both stressors were observed, suggesting that
362 contamination by hydrocarbons impacts fish metabolism when exposed to normoxia, but no
363 additional effects were observed under hypoxia. However, such an interpretation should be
364 cautiously treated, bearing in mind this study investigated the combined effect of hypoxia and
365 hydrocarbons on a single species, at one hypoxia level. Additionally, our experiments dictated
366 that fish were subjected to specific conditions, such as hypoxia duration and the sequential
367 application of stressors (i.e. fish were contaminated and then exposed to hypoxic conditions).
368 Indeed, the combined effects of hypoxia and hydrocarbons might have been different if
369 contamination had occurred during hypoxia.

370 Therefore, further investigations are necessary to determine the combined effects of both
371 stressors that coexist worldwide. For instance, future studies could investigate how
372 hydrocarbons modify oxygen threshold values below which fish metabolism is impacted.
373 Furthermore, studies on physiological functions linked to animal fitness (e.g. reproduction,

374 sexual behaviour) would increase our knowledge on the effects of these combined stressors
375 on fish and aquatic organisms. Several other interactions could be investigated, such as
376 temperature with contaminants, especially within the current marine heat wave context.

377

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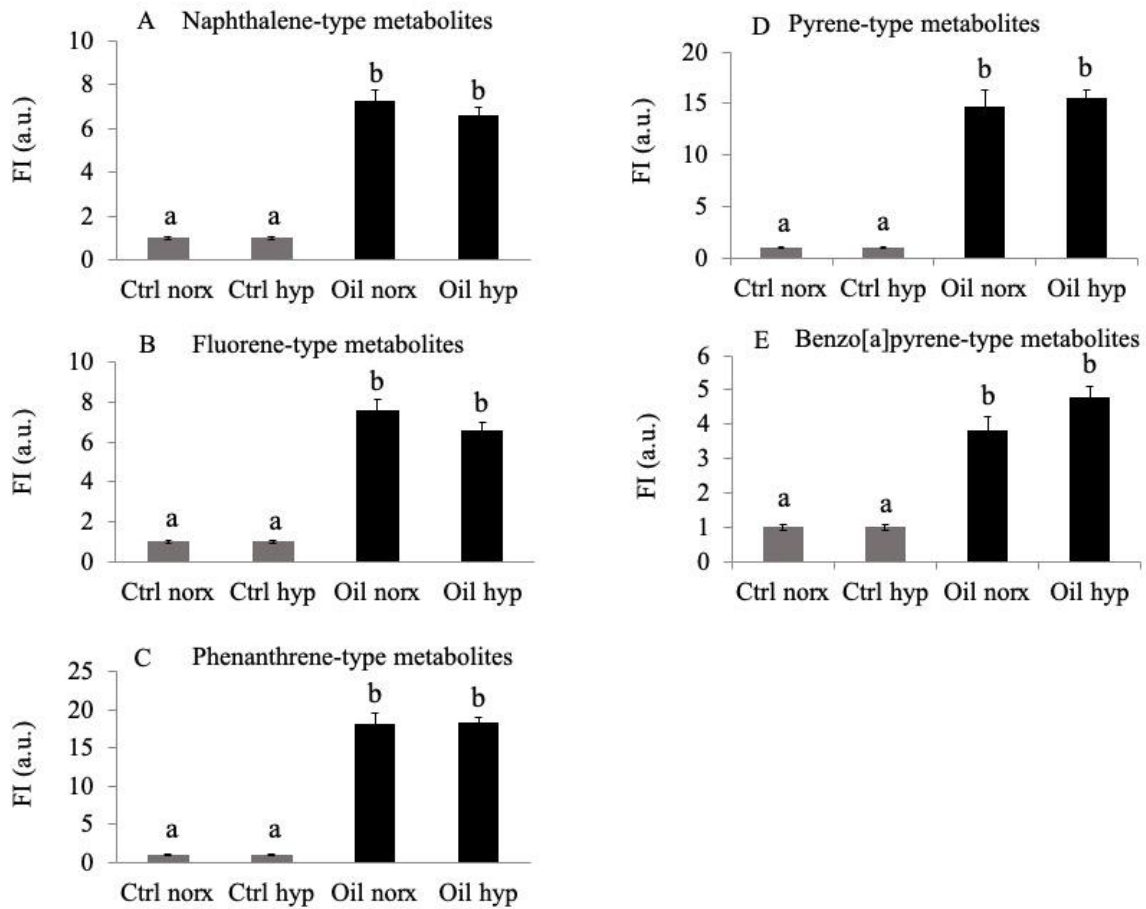
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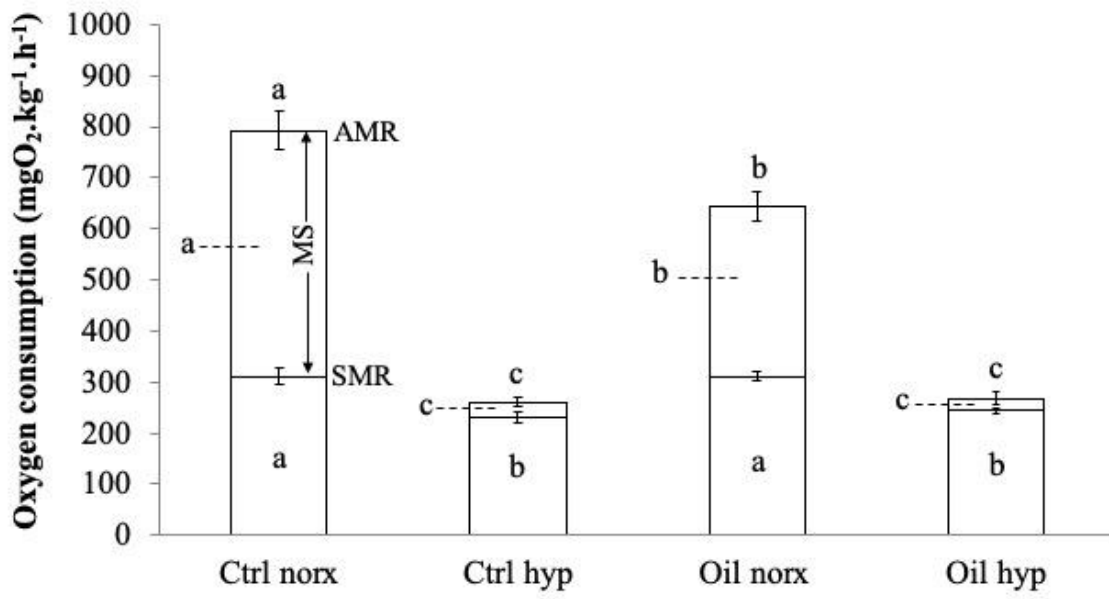
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561 **Figure 1.** The relative concentrations of polycyclic aromatic hydrocarbon (PAH) metabolites
562 in fish bile, expressed in fluorescence intensity units, for each fish group: 1) Ctrl norx are sea
563 bass previously exposed to a control treatment, and normoxic conditions. 2) Ctrl hyp are fish
564 exposed to a control treatment, and hypoxic conditions. 3) Oil norx are fish exposed to oil,
565 and normoxic conditions. 4) Oil hyp are fish exposed to oil, under hypoxic conditions.
566 Naphthalene, fluorene, phenanthrene, pyrene, benzo[a]pyrene type derived metabolites were
567 measured using fixed wavelength fluorescence (FF) at emission/excitation wavelengths of
568 290 nm/335 nm, 275 nm/328 nm, 250 nm/370 nm, 341 nm/383 nm and 380 nm/430 nm,
569 respectively. All are represented in histograms A, B, C, D, E. Values represent means \pm
570 standard errors of 11 individuals. Different letters above bars indicate significant differences,
571 where $P < 0.05$. Two-way ANOVA was used for statistical analyses. FI = fluorescence
572 intensity and a.u. = arbitrary units.
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576 **Figure 2.** Active metabolic rate (AMR) represents the standard metabolic rate (SMR; lower
577 parts of the bars) plus the metabolic scope (MS; upper parts of the bars) 1) Ctrl norx are sea
578 bass previously exposed to a control treatment, and normoxic conditions. 2) Ctrl hyp are fish
579 exposed to a control treatment, and hypoxic conditions. 3) Oil norx are fish exposed to oil,
580 and normoxic conditions. 4) Oil hyp are fish exposed to oil, under hypoxic conditions. Results
581 are expressed in $\text{mg O}_2 \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ and represent mean values (\pm standard errors) for 11
582 individuals. Different letters above bars indicate a significant difference ($P < 0.05$) between
583 the AMR means obtained for different experimental conditions. Different letters in lower
584 parts of the bars indicate a significant difference ($P < 0.05$) between the SMR means.
585 Different letters in the upper parts of the bars indicate a significant difference ($P < 0.05$)
586 between MS means. Two-way ANOVA was used for statistical analyses.
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