

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

Thomas Milinkovitch, Stefano Marras, Fabio Antognarelli, Christel Lefrançois, Stéphane Le Floch, Paolo Domenici

## ▶ To cite this version:

Thomas Milinkovitch, Stefano Marras, Fabio Antognarelli, Christel Lefrançois, Stéphane Le Floch, et al.. The effects of hypoxia on aerobic metabolism in oil-contaminated sea bass (Dicentrarchus labrax). Chemosphere, 2020, 253, pp.126678. 10.1016/j.chemosphere.2020.126678. hal-03544287

## HAL Id: hal-03544287 https://hal.science/hal-03544287

Submitted on 26 Jan 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

1	The effects of hypoxia on aerobic metabolism in oil-contaminated
2	sea bass (Dicentrarchus labrax)
3	
4	Thomas Milinkovitch <sup>1</sup> , Stefano Marras <sup>1</sup> , Fabio Antognarelli <sup>1</sup> , Christel Lefrançois <sup>2</sup> ,
5	Stéphane Le Floch <sup>3</sup> , Paolo Domenici <sup>1</sup>
6	
7	Affiliations:
8	<sup>1</sup> CNR-IAS, Istituto per lo studio degli impatti Antropici e Sostenibilità in ambiente marino,
9	Località Sa Mardini, 09170 Torregrande (Oristano), Italy.
10	<sup>2</sup> LIENSs UMR 7266, Littoral Environnement et sociétés, La Rochelle 17000, France.
11	<sup>3</sup> Centre de Documentation de Recherche et d'Expérimentations sur les Pollutions
12	Accidentelles des Eaux (CEDRE), 715 rue Alain Colas, CS41836-F-29218 Brest Cedex 2,
13	France.
14	
15	Email for correspondence: t.milinkovitch@hotmail.fr
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

polycyclic aromatic hydrocarbons (PAHs) was estimated by evaluating relative 25 concentrations of bile metabolites. In terms of bile metabolites, our results validated the 26 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 normoxic waters, and that oil does not magnify hypoxia-induced effects on fish metabolism. 36

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

The northern Gulf of Mexico is a well-known example of hypoxia and petrogenic hydrocarbon coexistence. Indeed, while organisms are commonly exposed to hypoxia, thanks to nutrient discharge from the Mississippi and Atchafalaya Rivers (Diaz and Rosenberg, 2008), they also face petroleum contamination due to oil spills, such as Deep Water Horizon (White *et al.*, 2012) and operational discharges (Wang *et al.*, 2014). In terms of the scientific literature on this subject, evaluating the combined effects of hypoxia and oil is still a new area of research, where more investigation is required. Nonetheless, limited studies are available and have investigated the physiological effects of both stressors in teleost fish (Negreiros *et al.*, 2011; Claireaux *et al.*, 2013; Dasgupta *et al.*, 2015; Mager *et al.*, 2018; Milinkovitch *et al.*, 2019). Among them, some of these studies have challenged organisms with severe levels of hypoxia and/or contamination, sporadically encountered on the field (Claireaux *et al.*, 2013; Dasgupta *et al.*, 2015). Others assessed biological effects at sub-individual levels (e.g. Negreiros *et al.*, 2011), consequently, impact predictions on organism fitness are uncertain.

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 metabolic rate (SMR), and was chosen as a biological parameter of interest, since it estimates 86 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** 

#### 100 **2.1. Animals**

101 Forty-four juvenile sea bass, aged 1.5-2 years old were used. Fish were provided by Aquanord Ichtus (Gravelines, France). Sea bass were acclimated in 2 m<sup>3</sup> tanks, with an open 102 103 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,  $35.30 \pm 0.06\%$ ,  $21.28 \pm 0.09$ °C and  $7.96 \pm 0.02$ , 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  $\pm$  0.13 cm and 35.27  $\pm$  0.88 g, respectively.

109

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

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

115

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

122

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

treatments, and two fish were exposed to the control treatment (four fish exposed per day). In total, we carried out eleven days of exposure; 22 fish were exposed to oil treatments, and 22 fish were exposed to control treatments. During the exposure period, temperature, salinity, pH and dissolved oxygen were stable:  $21.30^{\circ}C \pm 0.10$ ,  $35.06 \pm 0.10\%$ ,  $8.06 \pm 0.01$ ,  $99.60 \pm 0.40$ % 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 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 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 ( $21.50 \pm 0.06^{\circ}$ 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 mg  $O_2/L$ ). 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 mg O<sub>2</sub>/L), resulting in homogenous concentrations between 24-26% air 162 saturation (1.72 mg O<sub>2</sub>/L and 1.87 mg O<sub>2</sub>/L, respectively). Oxygen reduction was performed during 3 h to generate a progressive oxygen decline (Lefrançois and Domenici, 2006), to 163 164 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 appropriate software (Pre-Sens Oxy 4v2). Oxygen consumption was calculated using linear 171 172 least-squares regression (excluding the first and last 2 min of each 15 min closed phase) and expressed as mg of  $O_2$ .  $h^{-1}$ .kg<sup>-1</sup>. Then, to integrate the log-log allometric relationship between 173

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

176

177 Fish were left in the respirometer for 24 h, and oxygen consumption was measured every 30 min. SMR was evaluated after 20 h, using the 15<sup>th</sup> percentile method (Chabot and Claireaux, 178 179 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**

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

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

202

#### 203 **2.5. Statistical analysis**

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

211

#### **3. Results**

#### 213 **3.1. Hydrocarbon exposure**

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

218

ANOVA results showed that oil significantly affected the relative concentrations of all PAH types (i.e. naphtalene, fluorene, phenanthrene, pyrene, benzo[a]pyrene) (P < 0.01; Figure 1). *Post hoc* tests demonstrated significantly (P < 0.01) higher concentrations of metabolites in contaminated fish (Oil norx and Oil hyp), when compared to uncontaminated fish (Ctrl norx and Ctrl hyp). Results showed that hypoxia did not affect the relative concentrations of PAH metabolite types since no significance was highlighted between Oil norx and Oil hyp groupsnor between Ctrl norx and Ctrl hyp groups.

- 226
- 227

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

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

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

237

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

242

### **4. Discussion**

244

## 4.1. TPHs concentrations and PAHs metabolites

Our results revealed TPH concentration stability in the tank of exposure during the 18 h exposure. In addition, TPH concentrations did not show any statistical differences between replicates, demonstrating repeatability of the contamination protocol. Our results also showed that fish exposed to oil treatment were contaminated with  $13.6 \pm 0.5$  mg/L and  $14.8 \pm 0.8$  mg/L oil, at T = 0 h and T = 18 h, respectively. This order of magnitude agreed with data from Lewis and Daling (2001) when modelling oil slick dispersion, and with measurements conducted in the field, during oil spills (Lunel, 1995). Moreover, it is interesting to note that ships of 400 gross tonnage and above are allowed to discharge oil contaminated water into the sea if concentration does not exceed 15 mg/L of seawater (Convention Marpol, 1973). Thus, the oil concentrations used in this study were appropriate for investigating the impact of oil spills and operational discharge.

In order to investigate the combined effects of oil and hypoxia, we first exposed fish to oil and then to hypoxia for 24h. Although a decrease of hydrocarbon body burden could operate during these 24h, we believe it was not substantial since Dussauze *et al.* (2015) showed that 14 days were required to observe an approximate 50% decrease in total PAHs in the muscle of *Dicentrarchus labrax*. Be that as it may, we measured relative biliary metabolite concentrations (at the end of the hypoxic/normoxic period) in order to ensure the differential 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 (Ramachandran et al., 2006), but we cannot exclude ingestion as a potential mode of 272 273 incorporation. Benzo[a]pyrene uptake was highly relevant since this PAH has been shown to

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

Our physiological data were in line with other studies. For example, Claireaux *et al.* (2006) measured AMR and SMR values of 408 and 177 mg of  $O_2$ . h<sup>-1</sup>.kg<sup>-1</sup>, respectively, for juvenile sea bass acclimated to 22°C in control conditions. Although these values were almost half the values observed in our study, they were in the same order of magnitude. This difference could be attributable to different sizes of fish tested and/or to our methodology, since we determined AMR and SMR by static respirometry, whereas Claireaux *et al.* (2006) used a swimming 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 japonicus) were exposed to 75% air saturation. In our study, we exposed fish to 25% air 298

saturation (1.80 mg O<sub>2</sub>/L at 21.5°C), which induced a substantial significant reduction (67%)
in AMR, thereby contributing to a drastic 94% reduction in MS. In accordance with Fry (Fry,
1947), such a strong reduction in MS prevents fish from conducting discretionary activities
(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 O<sub>2</sub>/ 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 O<sub>2</sub>/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  $O_2/L$  (42.9 % a.s.) over 42 days.

In relation to the impact of physically dispersed oil (normoxic conditions), our results revealed that this contamination induced a 20% decrease in AMR, and a consequent 32% decrease in MS. This data suggested a reduction in the metabolic energy required for discretionary activities. These results are in contrast to a previous study (Milinkovitch *et al.*, 2012), that showed no effects of physically dispersed oil on AMR in golden grey mullet (*Liza 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 hydrocarbon contamination used by Mager et al. (2018) was lower than our study, which 355 356 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  $\leq 1.8$  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.

Therefore, further investigations are necessary to determine the combined effects of both stressors that coexist worldwide. For instance, future studies could investigate how hydrocarbons modify oxygen threshold values below which fish metabolism is impacted. 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

## 378 Acknowledgements

- 379 We thank the European Commission, and especially Marie Curie Action for their financial
- 380 support of the 'DISHY' project, and the individual European postdoctoral research
- 381 fellowship.
- 382

## 383 **5. References**

Aas E, Baussant T, Balk L, Liewenborg B, Andersen OK (2000) PAH metabolites in bile,
cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil
exposure: a laboratory experiment with Atlantic cod. Aquat. Toxicol. 51, 241–258.

387

Altieri AH, Harrison SB, Seemann J, Collin R, Diaz RJ, Knowlton N, 2017. Tropical dead
 zones and mass mortalities on coral reefs. PNAS 114, 3660-3665

Agamy E, 2013. Sub chronic exposure to crude oil, dispersed oil and dispersant induces
histopathological alterations in the gills of the juvenile rabbit fish (*Siganus canaliculatus*).
Ecotoxicol. Environ. Saf. 92, 180–190

- Amiard-Triquet C, Amiard JC, 2008. Les biomarqueurs dans l'évaluation de l'état écologique
   des milieux aquatiques. Lavoisier Tec et Doc, Paris
- Bejarano AC, Michel J, 2016. Oil spills and their impacts on sand beach invertebrate communities: a literature review. Environ. Pol. 218, 709-722
- Breitburg D, Levin LA, Oschlies A, Grégoire M, Chavez FP, Conley DJ, Garçon V,
  Gilbert D, Gutiérrez D, Isensee K, Jacinto GS, Limburg KE, Montes I, Naqvi SWA, Pitcher
  GC, Rabalais NN, Roman MR, Rose KA, Seibel BA, Telszewski M, Yasuhara M, Zhang J,
- 400 2018. Declining oxygen in the global ocean and coastal waters. Science. 359, 6371
- 401 Chabot D, Claireaux G, 2008. Environmental hypoxia as a metabolic constraint on fish: the 402 case of Atlantic cod, *Gadus morhua*. Mar. Poll. Bull. 57, 287–294.
- 403 Claireaux G, Lagardère JP, 1999. Influence of temperature, oxygen and salinity on the 404 metabolism of the European sea bass. J. Sea Res. 42, 157-168.

- Claireaux G, Webber DM, Lagardère JP, Kerr SR, 2000. Influence of water temperature and
  oxygenation on the aerobic metabolic scope of Atlantic cod (*Gadus morhua*). J. Sea Res. 44,
  257-265
- Claireaux, Couturier C, Groison AL, 2006. Effect of temperature on maximum swimming
  speed and cost of transport in juvenile European sea bass (*Dicentrarchus labrax*). J. Exp.
  Biol. 209, 3420-3428
- 411 Claireaux G, Lefrançois C, 2007. Linking environmental variability and fish performance.
- 412 integration through the concept of metabolic scope for activity. Philos. Trans. R. Soc Lond. B:
- 413 Biol. Sci. 362, 2031–2041
- 414 Claireaux G, Theron M, Prineau M, Dussauze M, Merlin FX, Le Floch S, 2013. Effects of oil 415 exposure and dispersant use upon environmental adaptation performance and fitness in the
- 416 european sea bass, *Dicentrarchus labrax*. Aquat. Toxicol. 131, 160-170.
- 417 Claireaux G, Chabot D, 2016. Responses by fishes to environmental hypoxia: integration
  418 through Fry's concept of aerobic metabolic scope. J. Fish Biol. 88, 232-251
- 419 Claireaux G, Queau P, Marras S, Le Floch S, Farrell AP, Nicolas-Kopec A, Lemaire P,
- 420 Domenici P, 2018. Avoidance threshold to oil water-soluble fraction by a juvenile marine
- 421 teleost fish. Environ. Toxicol. Chem. 37, 854-859.
- 422 Cohen A, Nugegoda D, Gagnon MM, 2001. Metabolic Responses of Fish Following
  423 Exposure to Two Different Oil Spill Remediation Techniques. Ecotox. Environ. Saf. 48, 306424 310
- 425 Costanza R, d'Arge R, de Groot R, Farber S, Grasso M, Hannon B, Limburg K, Naeem S,
  426 O'Neill RV, Paruelo J, Raskin RG, Sutton P, Van den Belt M, 1997. The value of the world's
  427 ecosystem services and natural capital. Nature 387, 253-260.
- 428 Dasgupta S, Huang IJ, McElroy AE, 2015. Hypoxia enhances the toxicity of cor- exit 429 EC9500A and chemically dispersed southern Louisiana sweet crude oil (MC-242) to 430 sheepshead minnow (*Cyprinodon variegatus*) larvae. PLOS ONE. 10, 1-9
- 431 Diaz RJ, Rosenberg R, 1995. Marine benthic hypoxia: a review of its ecological effects and
  432 the behavioural responses of benthic macrofauna. Oceanogr. Mar. Biol. Annu. Rev. 33, 245433 303
- 434 Diaz RK, Rosenberg R, 2008. Spreading dead zones and consequences for marine 435 ecosystems. Science 321, 926-929.
- 436 Domenici P, Lefrançois C, Shingles A, 2007. Hypoxia and the antipredator behaviours of 437 fishes. Phil. Trans. Royal Soc. 362, 2105-2121.
- 438 Dussauze M, Danion M, Le Floch S, Lemaire P, Pichavant-Rafini K, Theron M, 2015. Innate
  439 immunity and antioxidant systems in different tissues of sea bass (*Dicentrarchus labrax*)
  440 exposed to crude oil dispersed mechanically or chemically with Corexit 9500. Ecotox.
  441 Environ Sof 120, 270, 278
- 441 Environ. Saf. 120, 270-278.

- 442 FAO, 2018 Fisheries statistics and information. In: FAO Fisheries and Aquaculture
  443 Department. FAO, Rome. http://www.fao.org/ fishery/statistics/en
- Farrell AP, Richards JG, 2009. Defining Hypoxia: An Integrative Synthesis of the Responses
   of Fish to Hypoxia. Fish physiology, 27, 487-503
- Farrell AP, 2011. Encyclopedia of fish physiology: from genome to environment academicpress, Ottawa, Canada.
- Fitzgibbon QP, Strawbridgeb A, Seymour RS, 2007. Metabolic scope, swimming
  performance and the effects of hypoxia in the mulloway, *Argyrosomus japonicus (Pisces: Sciaenidae*). Aquaculture 270, 358–368
- Fox J, White P, Spassova M, Flowers L, 2013. Toxicological Review of Benzo[a]pyrene.
   EPA/635/R-17/003Fa
- 453 Fry FE, 1947. Effects of the environment on animal activity. Ontario Fisheries Research454 Laboratory Publication, Biol. Ser. 55, 1-62.
- Fusey P, Oudot J, 1976. Comparaison de deux méthodes d'évaluation de la biodégradation des
  hydrocarbures in vitro. Mater Org. (Berlin) 4, 241–51.
- Gagnon MM, Holdway DA, 1999. Metabolic Enzyme Activities in Fish Gills as Biomarkers
  of Exposure to Petroleum Hydrocarbons. Ecotox. Environ. Saf. 44, 92-99
- Geraudie P, Bakkemo R, Milinkovitch T, Thomas-Guyon H, 2016. First evidence of marine
  diesel effects on biomarker responses in the Icelandic scallops, *Chlamys islandica*. Environ.
  Sci. Pol. Res. 23, 504-512
- 462 GESAMP, 2009. Pollution in the open oceans: a review of assessments and related studies.
  463 IMO/FAO/UNESCO-IOC/UNIDO/WMO/IAEA/UN/UNEP Joint Group of Experts on the
  464 Scientific Aspects of Marine Environmental Protection. 79, 1-96.
- 465 GESAMP, 2015. Pollution in the Open Oceans. IMO/FAO/UNESCO466 IOC/UNIDO/WMO/IAEA/UN/UNEP Joint Group of Experts on the Scientific Aspects of
  467 Marine Environmental Protection. 91, 1-87
- Harriss R, 2016. Arctic Offshore Oil: Great Risks in an Evolving Ocean. Environ. : Sci.
  Policy. Sustain. Develop. 58, 1-10
- Hesni MA, Savari A, Sohrab AD, Mortazavi MS, 2011. Gill Histopathological Changes in
  Milkfish (Chanos chanos) Exposed to Acute Toxicity of Diesel Oil. World App. Sci. J. 14,
  1487-1492
- Jeppesen R, Rodriguez M, Rinde J, Haskins J, Hugues B, Mehner L, Wasson K, 2018. Effects
  of Hypoxia on Fish Survival and Oyster Growth in a Highly Eutrophic Estuary. Estuar. Coast.
  475 41, 89-98

- 476 Jourdan-Pineau H, Dupont- Prinet A, Claireaux G, McKenzie DJ, 2009.-An Investigation of
- 477 Metabolic Prioritization in the European Sea Bass, *Dicentrarchus labrax*. Physiological and
- 478 Biochemical Zoology. 83, 68-77
- Kopecka-Pilarczyk J, Correia AD, 2009. Biochemical response in gilthead seabream (*Sparus aurata*) to in vivo exposure topyrene and fluorene. J. Exp. Mar. Biol. Ecol. 372, 49–57
- 481 Lefrançois C, Claireaux G, 2003. Influence of ambient oxygenation and tempperature on
   482 metabolic scope and scope for heart rate in the Common sole *Solea solea*. Mar. Ecol. progress
- 483 series, 259, 273-284.
- Lefrançois C, Domenici P, 2006. Locomotor kinematics and behaviour in the escape response
  of European sea bass, *Dicentrarchus labrax* L., exposed to hypoxia. Mar. Biol. 149, 969-977.
- 486 Lefrançois C, Shingles A, Domenici P, 2005. The effect of hypoxia on locomotor 487 performance and behaviour during escape in *Liza aurata*. J. Fish Biol. 67, 1711-1729.
- Lewis A, Daling P, 2001. Oil spill dispersants. (ed A. L. O. S. Consultant), pp. 28. SINTEF,
  Staines.
- 490 Lunel T, 1995. The Braer oil spill: oil fate governed by dispersion. Proceedings of the 1995491 International Oil Spill Conference, Long Beach, California, USA
- McNatt RA, Rice JA, 2004. Hypoxia-induced growth rate reduction in two juvenile estuarydependent fishes. J. Exp. Mar. Biol. 311, 147-156-
- Mager EM, Esbaugh AJ, Stieglitz JD, Hoenig R, Bodinier C, Incardona JP, Scholz
  NL, Benetti DD, Grosell M, 2014. Acute Embryonic or Juvenile Exposure to *Deepwater Horizon* Crude Oil Impairs the Swimming Performance of Mahi-Mahi (*Coryphaena hippurus*). Environ. Sci. Technol. 48, 7053-7061
- Mager EM, Pasparakis C, Stieglitz JD, Hoenig R, Morris JM, Benettia DD, Grosella M, 2018.
  Combined effects of hypoxia or elevated temperature and Deepwater Horizon crude oil
  exposure on juvenile mahi-mahi swimming performance. Mar. Environ. Res. 139, 129-135.
- MARPOL, 1973. Annex 1 -Regulations for preventing oil pollution from ships. Chapter 3 Requirements for machinery spaces of all ships. Part C Control of operational discharge of
   oil
- Milinkovitch T, Sanchez W, Ndiaye A, Le Floch S, Thomas-Guyon H, 2011a. Liver antioxidant responses and innate immune function in golden grey mullet (*Liza aurata*) following exposure to dispersed crude oil. Aquat.Toxicol. 101, 155–164.
- Milinkovitch T, Godefroy J, Théron M, Thomas-Guyon H, 2011b. Toxicity of dispersant
  application: biomarkers responses in gills of juvenile golden grey mullet (*Liza aurata*).
  Environ. Pol. 159, 2921–2928.
- 510 Milinkovitch T, Lucas J, Le Floch S, Thomas-Guyon H, Lefrançois C (2012) Effect of 511 dispersed crude oil exposure upon the aerobic metabolic scope in juvenile golden grey mullet 512 (*Liza aurata*). Mar. Pol. Bul. 64, 865–871.

- 513 Milinkovitch T, Geraudie P, Camus L, Huet V, Thomas-Guyon H, 2015. Biomarker 514 modulation associated with marine diesel contamination in the Iceland scallop (Chlamys 515 islandica). Environ. Sci. Pol. Res. 22, 19292-19296
- Milinkovitch, T., Antognarelli, F., Lacroix, C., Marras, S., Satta, A., Le Floch, S., Domenici,
  P., 2019. The effect of hypoxia and hydrocarbons on the anti-predator performance of
  European sea bass (*Dicentrarchus labrax*). Environ. Pollut. 251, 581-590
- Nakano T, Yamada K, Okamura K, 2017. Duration rather than frequency of hypoxia causes
  mass mortality in ark shells (*Anadara kagoshimensis*). Mar. Pol. Bull. 125, 86-91
- Neff JM, 2002. Polycyclic aromatic hydrocarbons in the aquatic environment: sources, fateand biological effects. Applied Science publishers, London
- Negreiros LA, Silva BF, Paulino MG, Fernandes MN, Chippari-Gomes AR, 2011. Effects of
  hypoxia and petroleum on the genotoxic and morphological parameters of Hippocampus reidi.
  Comp. Biochem. Physiol., C 153, 408-414.
- 526 Pichavant K, Person-le-Ruyet J, Le Bayon N, Sévère A, Leroux A, Quéméner L, Maxime V,
- 527 2000. Effects of hypoxia on growth and metabolism of juvenile turbot. Aquacult. 188, 103-528 114
- Ramachandran SD, Sweezey MJ, Hodson PV, Boudreau M, Courtenay SC, Lee K, King T,
  Dixon JA, 2006. Influence of salinity and fish species on PAH uptake from dispersed crude
  oil. Mar. Poll. Bull. 52, 1182–1189
- Roche DG, Binning SA, Bosiger Y, Johansen JL, Rummer J.L., 2013. Finding the best
  estimates of metabolic rates in a coral reef fish. J. Exp. Biol. 216, 2103–2110.
- Rosewarne PJ, Wilson JM, Svendsen JC, 2016. Measuring maximum and standard metabolic
  rates using intermittent-flow respirometry: a student laboratory investigation of aerobic
  metabolic scope and environmental hypoxia in aquatic breathers. J. Fish Biol. 88, 265-283
- Selman M, Greenhalgh S, Diaz R, Sugg Z, 2008. Eutrophication and hypoxia in coastal
  areas : a global assessment of the state of knowledge. World ressources institute policy note,
  1, 1-6
- 540 Shearer C, 2018. Decreasing supplies, increasing risks in oil development *in* The oxford 541 handbook of energy and society. Oxford university press.
- 542 Szlinder-Richert J, Nermer T, Szatkowska U (2014) PAH metabolites in European eels
   543 (*Anguilla anguilla*) as indicators of PAH exposure: Different methodological approaches
- 544
- 545 Terova G, Rimoldi S, Cora S, Bernardini G, Gornati R, Saroglia M, 2008. Acute and chronic
- 546 hypoxia affects HIF-1a mRNA levels in sea bass (Dicentrarchus labrax). Aquaculture 279,
- 547 150-159.

Wang Z, Liu Z, Xu K, Mayer LM, Zhang Z, Kolker AS, Wu W, 2014. Concentrations and
sources of polycyclic aromatic hydrocarbons in surface coastal sediments of the northern Gulf
of Mexico. Geochem. Transac. 15, 1-12

551 White HK, Hsing PY, Cho W, Shank TM, Cordes EE, Quattrini AM, Nelson RK, Camilli 552 R, Demopoulos AWJ, German CR, Brooks JM, Roberts HH, Shedd W, Reddy CM, Fisher 553 CR, 2012. Impact of the *Deepwater Horizon* oil spill on a deep-water coral community in the 554 Gulf of Mexico. PNAS. 109, 20303-20308

- 555 Worm B, Barbier EB, Beaumont N, Duffy JE, Folke C, Halpern BS, Jackson JBC, Lotze HK,
- 556 Micheli F, Palumbi SR, Sala E, Selkoe KA, Stachowicz JJ, Watson R (2006). Impacts of
- 557 Biodiversity Loss on Ocean Ecosystem Services. Science. 314, 787-790.
- 558
- 559
- 560

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 exposed to a control treatment, and hypoxic conditions. 3) Oil norx are fish exposed to oil, 564 and normoxic conditions. 4) Oil hyp are fish exposed to oil, under hypoxic conditions. 565 566 Naphthalene, fluorene, phenanthrene, pyrene, benzo[a]pyrene type derived metabolites were measured using fixed wavelength fluorescence (FF) at emission/excitation wavelengths of 567 290 nm/335 nm, 275 nm/328 nm, 250 nm/370 nm, 341 nm/383 nm and 380 nm/430 nm, 568 569 respectively. All are represented in histograms A, B, C, D, E. Values represent means ± 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.



#### 574 575

576 Figure 2. Active metabolic rate (AMR) represents the standard metabolic rate (SMR; lower parts of the bars) plus the metabolic scope (MS; upper parts of the bars 1) Ctrl norx are sea 577 bass previously exposed to a control treatment, and normoxic conditions. 2) Ctrl hyp are fish 578 579 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 are expressed in mg  $O_2$ .kg<sup>-1</sup>.h<sup>-1</sup> and represent mean values (± standard errors) for 11 581 582 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 parts of the bars indicate a significant difference (P < 0.05) between the SMR means. 584 585 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.



