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

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Abstract

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

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

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

1. Introduction

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

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

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

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.

Therefore, we sought to experimentally evaluate the effects of hypoxia on the metabolic scope (MS) of the European Sea bass (*Dicentrarchus labrax*), previously contaminated with oil. MS 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 the instantaneous energy an organisms allocates to demanding activities (i.e. locomotion, digestion, feeding, etc.), and is therefore deemed an optimal, measurable fitness proxy (Claireaux and Lefrançois, 2007). Moreover, MS is modulated by hypoxic environments (Farrell and Richards, 2009) and oil exposure (Mager *et al.*, 2014), but no studies have investigated the cumulative effects of both stressors on sea bass metabolism. Sea bass (*Dicentrarchus labrax*) was chosen as a biological model thanks to several key characteristics; its primary role as a marine predator, its economic and commercial relevance (aquaculture production of 165, 915 tons and capture fisheries of 6,919 tons in 2016; FAO, 2018), and its perceived sensitivity to hypoxia (Teroiva, 2008) and hydrocarbons (Claireaux *et al.*, 2018).

2. Materials and Methods

2.1. Animals

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

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.

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).

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: $21.30^{\circ}\text{C} \pm 0.10$, $35.06 \pm 0.10\text{‰}$, 8.06 ± 0.01 , 99.60 ± 0.40 % air saturation, respectively.

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

2.3. Respirometry measurements under hypoxic or normoxic conditions

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

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

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

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).

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

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).

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).

3. Results

3.1. Hydrocarbon exposure

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 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.

ANOVA results showed that oil significantly affected the relative concentrations of all PAH types (i.e. naphthalene, 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 groups nor between Ctrl norx and Ctrl hyp groups.

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).

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).

4. Discussion

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 ± 0.5 mg/L and 14.8 ± 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.

Our results showed that all types of metabolites were significantly higher in the bile from fish exposed to oil treatment, when compared to control fish. This validates the uptake by organisms of all types of PAHs studied (i.e. naphthalene, fluorene, phenanthrene, pyrene, benzo[a]pyrene). Bile metabolite incorporation of light PAHs was expected, since these compounds are highly soluble in seawater, and are likely to be incorporated into aquatic organisms by diffusion (Neff, 2002). In contrast, the observed incorporation of heavier and the more hydrophobic PAHs, such as benzo[a]pyrene, did not occur by similar processes, since these compounds have a low solubility in seawater (Neff, 2002). Most likely, 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 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.

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 O₂. h⁻¹.kg⁻¹, 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.

Our results initially showed an AMR decrease under hypoxic conditions, which was in agreement with previous studies for teleost fish (Claireaux and Lagardère, 1999; Lefrançois and Claireaux, 2003; Jourdan-Pineau *et al.*, 2009). Indeed, while some compensatory mechanisms exist (e.g. hyperventilation), fish cannot maintain optimal oxygen extraction from seawater, when facing hypoxic conditions (Farrell, 2011). As a consequence, AMR is reduced and other aerobic performances, such as locomotion, are influenced by such reductions (Farrell, 2011). Several studies have observed that moderate hypoxia induces AMR reductions in teleost fish: e.g., Lefrançois *et al.* (2003) and Fitzgibbon *et al.* (2007) 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

saturation (1.80 mg O₂/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.).

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

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

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

contamination may not be a limiting factor. On this basis, it would be interesting to test the effects of hydrocarbons at moderate degrees of hypoxia (i.e. when respiration is not intensively reduced, and hydrocarbons are a limiting factor) to highlight the synergistic or cumulative effects of both stressors in these environments. Mager *et al.* (2018) showed a similar effect of hypoxia alone or combined with oil, on the critical swimming speed of Mahi-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 could explain the lack of effect of this contaminant.

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

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

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Figure 1. The relative concentrations of polycyclic aromatic hydrocarbon (PAH) metabolites in fish bile, expressed in fluorescence intensity units, for each fish group: 1) Ctrl norx are sea 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, and normoxic conditions. 4) Oil hyp are fish exposed to oil, under hypoxic conditions. Naphthalene, fluorene, phenanthrene, pyrene, benzo[a]pyrene type derived metabolites were measured using fixed wavelength fluorescence (FF) at emission/excitation wavelengths of 290 nm/335 nm, 275 nm/328 nm, 250 nm/370 nm, 341 nm/383 nm and 380 nm/430 nm, respectively. All are represented in histograms A, B, C, D, E. Values represent means \pm standard errors of 11 individuals. Different letters above bars indicate significant differences, where $P < 0.05$. Two-way ANOVA was used for statistical analyses. FI = fluorescence intensity and a.u. = arbitrary units.

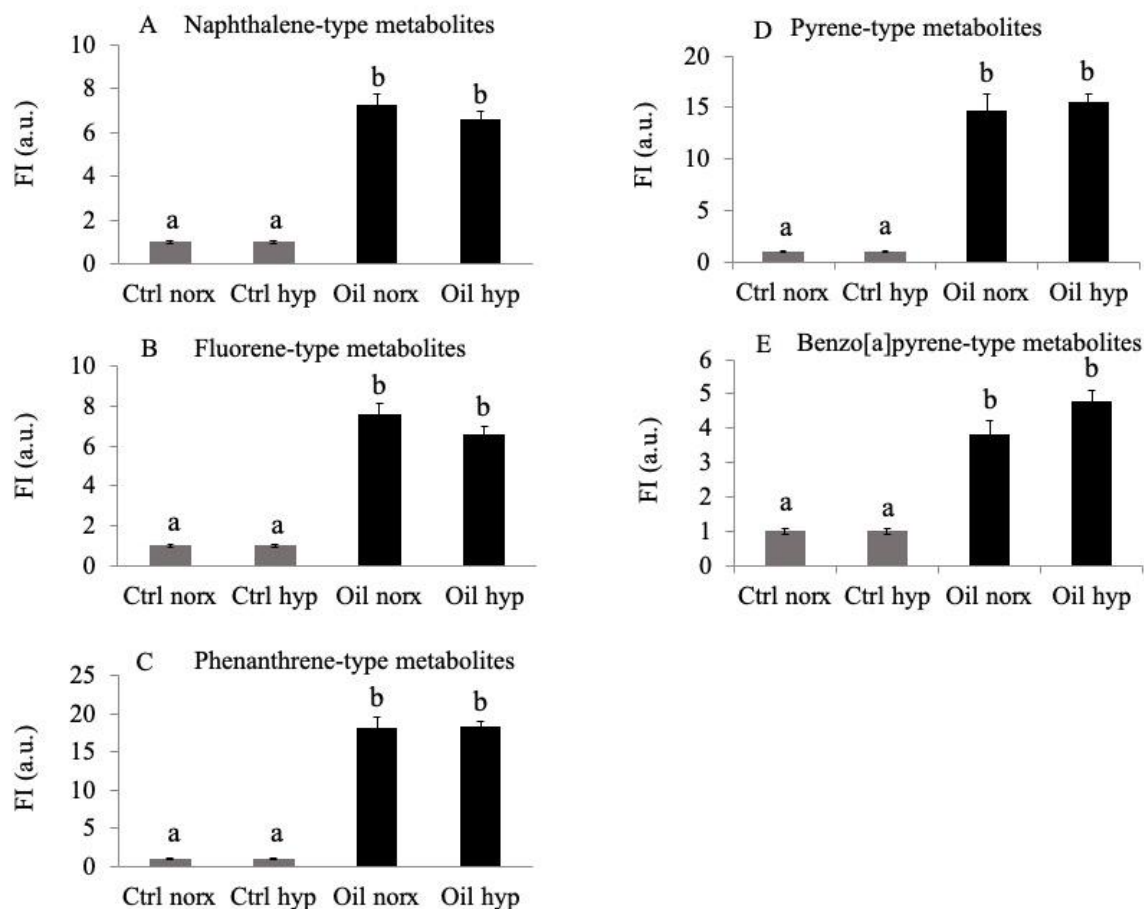
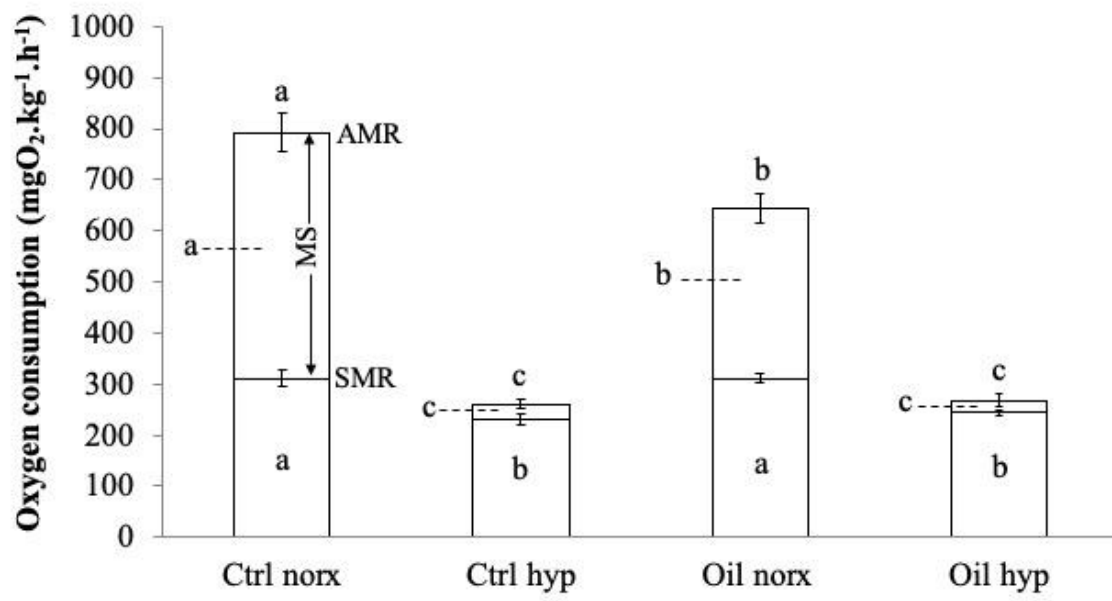


Figure 2. Active metabolic rate (AMR) represents the standard metabolic rate (SMR; lower parts of the bars) plus the metabolic scope (MS; upper parts of the bars) 1) Ctrl norx are sea bass previously exposed to a control treatment, and normoxic conditions. 2) Ctrl hyp are fish exposed to a control treatment, and hypoxic conditions. 3) Oil norx are fish exposed to oil, and normoxic conditions. 4) Oil hyp are fish exposed to oil, under hypoxic conditions. Results are expressed in $\text{mg O}_2 \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ and represent mean values (\pm standard errors) for 11 individuals. Different letters above bars indicate a significant difference ($P < 0.05$) between 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. Different letters in the upper parts of the bars indicate a significant difference ($P < 0.05$) between MS means. Two-way ANOVA was used for statistical analyses.



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