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1 **Combined effects of high hydrostatic pressure and dispersed oil on**
2 **the metabolism and the mortality of turbot hepatocytes**
3 **(*Scophthalmus maximus*)**

4

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16

17

18 **Abstract**

19 Since the DeepWater Horizon oil spill and the use at 1450m depth of dispersant as a
20 technical response, the need of relevant ecotoxicological data on deep-sea
21 ecosystems becomes crucial. In this context, this study focused on the effect of high
22 hydrostatic pressure (10.1 MPa) on turbot hepatocytes isolated from fish exposed
23 either to chemically dispersed oil, mechanically dispersed oil or dispersant alone.
24 Potential combined effects of oil/dispersant and hydrostatic pressure, were assessed
25 on cell mortality (total cell death, necrosis and apoptosis), cell viability and on
26 hepatocyte oxygen consumption (MO_2). No change in cell mortality was observed in
27 any of the experimental conditions, whereas, the results of cell viability showed a
28 strong and significant increase in the two oil groups independently of the pressure
29 exposure. Finally, oil exposure and hydrostatic pressure have additive effects on
30 oxygen consumption at a cellular level. Presence of dispersant prevent any MO_2
31 increase in our experimental conditions. These mechanistic effects leading to this
32 increased energetic demand and its eventual inhibition by dispersant must be
33 investigated in further experiments.

34

35 **Keywords:** oil spill; dispersant; depth; hydrostatic pressure; cell toxicity; model fish

36

37 1. Introduction

38 Deep-seas above 1000m depth represent the large majority of the biosphere
39 (Jannasch & Taylor, 1984) and have been out of reach for human kind for centuries.
40 This situation is changing, the exploitation of deep-seas is now technically feasible
41 and starts to be economically relevant. In particular, offshore deep-water areas
42 represent an increasing part of the new oil exploration/production areas (Maddahi &
43 Mortazavi, 2011). Discharge of chemicals, whether chronic or accidental, could
44 upset the balance of these sensitive ecosystems. Therefore, there is a need to
45 increase available relevant data offering a better understanding of potential
46 environmental risks of anthropic activities in deep-sea areas (Mestre *et al.*, 2013).

47 The example of the *Deepwater Horizon* accident in the Gulf of Mexico in April
48 2010, one of the largest oil spills in the history, has been the most striking
49 demonstration of this need. During this accident, about 780 000 m³ of oil were
50 released into the environment at 1500 meters depth (Griffiths, 2012), and for the first
51 time, dispersant (7 000m³) was injected directly in the deep-sea environment
52 (Kujawinski *et al.*, 2011) to limit the rise of oil to the surface. Since dispersants are
53 designed in order to be used on an oil slick at the sea surface to stabilize the oil in
54 the water as a plume of droplets, their effects in deep-seas are clearly miss-
55 understood.

56 Additionally to in-field ecological studies, experimental approaches in
57 laboratory can provide relevant data to assess and understand the new field of deep-
58 sea ecotoxicology. Even if, to date, few data are available, several approaches can
59 be used to explore this new topic (Lemaire *et al.*, 2017). One of this approach is to
60 study the impact of contaminants at atmospheric pressure on piezotolerant species.

61 This type of study has already been carried out on three cold-water coral species:
62 *Paramuricea sp*, *Callogorgia delta* and *Leiopathes glaberrima* (DeLeo *et al.*, 2016) or
63 on a giant benthic amphipod *Eurythenes gryllus* (Olsen *et al.*, 2016). Nevertheless, in
64 order to have a better understanding of the potential impact of a chemical in deep-
65 sea environment, it is necessary to develop experimental protocols that reproduce as
66 closely as possible the preponderant abiotic parameters of this area, and in particular
67 the hydrostatic pressure (HP). The piezoecotoxicology also called ecobarotoxicology,
68 *i.e.* the use of hyperbaric chamber to assess the potential impact of chemicals at
69 simulated depth, offers a relevant way to understand this new domain of
70 ecotoxicology. This approach has been applied on several classes such as the
71 polychaete *Pomatoceros lamarcki* (Vevers *et al.*, 2010), the marine shrimp *Palaemon*
72 *varians* (Domingues, 2015), the mussel *Mytilus galloprovincialis* (Pinheiro *et al.*,
73 2019) or different fish species: *Pagellus bogaraveo* (Kopecka-Pilarczyk & Coimbra,
74 2010a); *Anguilla Anguilla* (Kopecka-Pilarczyk & Coimbra, 2010b); *Dicentrarchus*
75 *labrax* (Dussauze *et al.*, 2017; (Lemaire *et al.*, 2016) or *Scophthalmus maximus*
76 (Dussauze *et al.*, 2016) at pressures ranging from 10 to 40 MPa. An *in vitro*
77 approach has also been applied exposing liver slices of rock grenadier
78 *Coryphaenoides rupestris* to 0.1, 5 and 15 MPa (Lemaire *et al.*, 2012). However, as
79 far as we know, the use of isolated cells for piezoecotoxicological studies has not been
80 explored yet.

81 In this context, and in order to increase our understanding of the combined
82 effects of oil/dispersant and hydrostatic pressure, this study focused on the effect of a
83 10.1 MPa exposure on turbot hepatocytes isolated from fish exposed either to
84 chemically dispersed fuel oil, mechanically dispersed oil or dispersant alone. The
85 main objective of this study is to assess the effects of these chemicals on hepatocyte

86 oxygen consumption (MO_2) under high hydrostatic pressure. In order to be able to
87 link a potential modification of MO_2 with cellular mortality, cell viability was evaluated
88 using two classical methods after the pressure exposure.

89

90 **2. Materials and Methods**

91

92 This experiment followed the subsequent steps: 1/ animal contamination, 2/
93 hepatocyte isolation, 3/ cell exposure to high hydrostatic pressure and finally 4/
94 biological analyses.

95

96 **2.1 Fish contamination**

97

97 **2.1.1 Animals**

98 Juvenile turbot (*Scophthalmus maximus*, n= 32, weight from 150 to 200g) used in this
99 experiment were hatched in the aquaculture facility of *France turbot* (Trédarzec,
100 France). In the laboratory, fish were acclimatized in a 300 L seawater tank for two
101 weeks. Photoperiod was according to the season (12:12). Oxygen saturation (> 90
102 %), pH (8.10 ± 0.16) and temperature (maintained at 17°C) were measured daily.
103 Turbots were fed daily with dried commercial pellets provided by the hatchery. The
104 experiments have been validated by the local Ethic Committee and authorized by the
105 french "Ministère de l'Enseignement Supérieur et de la Recherche".

106

107 **2.1.2 Chemicals**

108 The petroleum oil used in the study was a crude oil from the Lula oil-field (25°30'0"S
109 43°30'0"W). This offshore oil field is located in Ultra Deep water at approximately

110 2000m depth. This oil contained 54% saturated hydrocarbons, 10% polar compounds
111 and 36% aromatic hydrocarbons. Finasol OSR 52, a commercial formulation from
112 TOTAL Fluides (Puteaux, France), was used as dispersant. It is a third generation oil-
113 based dispersant combining surfactants (amphiphilic molecules) and solvents.
114 Components of Finasol OSR52 were: docusate sodium 20-25%; Hydrocarbons, C1-
115 C14, n-alkanes, ioalkanes, cylics, <2%; aromatics 15-20%; (2-
116 methoxymethylethoxy)propanol 15-20%; Carboxylic acids, diC6-12 compounds, with
117 ethanolamine, boric acid compound with ethanolamine 0-2%; Ethanolamine 0-1%.

118

119 **2.1.3 Animal contamination**

120 Turbot contaminant exposure was realized in a high-density polyethylene tank of 50
121 liters following a method fully described in Milinkovitch *et al.* (2011). Each of these
122 tanks was equipped with a submersible pumping system allowing continuous water
123 homogenization.

124 After 24h without feeding, eight fishes were randomly allocated to each of the
125 following experimental conditions: control (C), mechanically dispersed oil (MD),
126 chemically dispersed oil (CD) and dispersant alone (D) and placed in the 50L
127 exposure tanks for 48 hours without water renewal.

128 In the case of the MD and CD conditions, 4g of oil were poured into 50L seawater
129 tanks (for a nominal concentration of 80mg/L), in the CD condition 0.2g of dispersant
130 were also added (dispersant / oil ratio: 1/20). For the D condition, 0.2g of dispersant
131 were added to 50L of seawater.

132 At the end of the 48h exposure, fishes were killed by cerebral dislocation. Livers and
133 gallbladders were then sampled. After their sampling, the gallbladders were frozen in

134 liquid nitrogen and stored at -80°C; the livers were extemporaneously used for cells
135 isolation.

136

137 **2.1.4 Measurements of Total Petroleum Hydrocarbon in seawater.**

138 Total petroleum hydrocarbon (TPH) concentration was measured after 12h, 24h, 36
139 and 48h during the exposure period for the four experimental conditions.

140 The total petroleum hydrocarbon concentrations were measured in triplicate.

141 Samples of 100mL of water were extracted three times with 10 mL of
142 dichloromethane (Carlo Erba Reactifs, SDS). The combined organic phases were
143 dried on anhydrous sulphate and the absorbance was measured at 390 nm (UVeVis
144 spectrophotometer, Unicam, France) as described by Fusey and Oudot (1976). The
145 results were expressed in mg/L, and the linearity of the response was checked
146 between 0.5 and 100 mg/L.

147

148 **2.1.5 Fixed wavelength fluorescence analysis of bile**

149 Gallbladders were used for semi-quantitative analysis of PAH biliary metabolites
150 (Vuorinen *et al.*, 2006). The bile was diluted in absolute ethanol (1/2000).

151 Measurements were performed with a Jasco FP-6200 (Tokyo, Japan). The
152 measurements were taken at two wavelengths pairs: 380:430nm characteristic of
153 benzo[a]pyrene type metabolites and 343:383nm characteristic of 1-hydroxy-pyrene
154 type metabolites. Values were expressed as arbitrary units (Lin *et al.*, 1996; Aas *et*
155 *al.*, 2000).

156

157 **2.2 Hepatocyte Isolation**

158 Cells were isolated by collagenase digestion as adapted from Olivier *et al.* (2006).
159 Briefly, a cannula was inserted into the hepatic portal vein and the liver perfused with
160 10 mL of a 320 mosm/kg standard isosmotic solution (152 mM NaCl, 3.4 mM KCl, 0.8
161 mM MgSO₄·7H₂O, 0.33 mM Na₂HPO₄·2H₂O, 0.44 mM KH₂PO₄, 5 mM NaHCO₃, 10
162 mM HEPES; pH 7.63) at a flow rate of 0.5 mL/min. The liver was then perfused with
163 the same volume of iso-osmotic solution enriched with 0.6 mM EDTA at 0.5 mL/min,
164 and finally with 15 mL of a collagenase solution (type IV, 1 mg/mL in iso-osmotic
165 solution) at 0.34 mL/min. The liver was scrapped off with a scalpel blade and filtered
166 through 200, 85 and 48 µm nylon mesh screens. Cells were centrifuged for 4 min at
167 500 rpm at 4 °C, and resuspended in 5 mL of iso-osmotic solution enriched with 5
168 mM glucose and 1.5 mM CaCl₂. Cells were maintained under gentle agitation at least
169 for 1 h at 17 °C prior to use.

170

171 **2.3 Cell exposure to high hydrostatic pressure**

172 **2.3.1 High Hydrostatic Pressure Exposure System**

173 The experimental system (previously described in Dussauze *et al.* 2016) consisted in
174 four PMMA hyperbaric tanks connected to a HPLC pump with PEEK tubing. These
175 tanks had in their center a 1.5 mL chamber where the cell suspension was
176 introduced. The homogenization of the chamber was performed thanks to a magnetic
177 stirring bar. The sealing of each chamber was realized with a rubber O-rings and a
178 screwed 4 mm thick PMMA cover. The temperature control of the system (at 17°C)
179 was ensured with a water circulation surrounding the body of the chamber. The
180 hydrostatic pressure was increased forcing isosmotic solution through an adjustable
181 counter-pressure generator. The hydrostatic pressure generated in the system was
182 recorded continuously with a pressure gauge.

183 **2.3.2 Protocol of cell exposure to HP**

184 Between 3 and 4 million cells (suspended in isosmotic solution) were placed into
185 each chamber well. The chambers were sealed, connected to the pressurizing
186 system and maintained 40 min at atmospheric pressure. During each experiment,
187 two chambers were kept at atmospheric pressure (0.1 MPa) and two chambers were
188 exposed to high hydrostatic pressure. In both pressurized chambers, the pressure
189 was increased at a speed of 1 MPa/min up to 10.1 MPa; the pressure was then
190 maintained 40 minutes and the chambers decompressed at 10 MPa/min.

191

192 **2.4 Biological analyses**

193 **2.4.1 Oxygen consumption measurement under high hydrostatic**
194 **pressure**

195 Oxygen partial pressures in the chamber well were recorded all along the
196 experimentation (as described Dussauze *et al.* 2016) using an oximeter Oxy-4
197 (Presens, Regensburg, Germany). The oxygen sensor spots were placed on the top
198 of the chamber well on the internal face of the PMMA covers.

199 The measurements were performed during the pressure exposure at 10 MPa, and
200 during the same time in the control 0.1 MPA chambers. After analysis of the protein
201 content of the cell suspension (Bradford method), the oxygen consumption was
202 expressed in $\mu\text{mol O}_2/\mu\text{g}$ of protein/min.

203

204 **2.4.2 Hepatocyte mortality**

205 The determination of total cell death, necrosis and apoptosis was performed thanks
206 to propidium iodide (PI) and annexin V – FITC staining (Miltenyi Biotec, 130-092-052

207 Kit) accordingly to the recommendations of use. After the pressure exposure in the
208 high-pressure chambers at 10.1 MPa (or after an equivalent stay in the control
209 chambers at atmospheric pressure), these analyses were performed in duplicate on
210 10^6 cells. Fluorescence was measured at two wavelengths pairs: 490/525nm for
211 annexin V – FITC and 535/617 nm for PI on a plate reader (SAFAS FLX-Xenius).
212 The measurement of cell viability was determined using the “Realtime-glo mt cell
213 viability” (from Promega) accordingly to the recommendations of use. In this kit, the
214 viability is indirectly evaluated via the reducing activity of the hepatocytes: the
215 reduction of an organic substrate coupled to a luciferase. Briefly, duplicates of
216 2.5×10^5 cells were placed in a 96-well photometric plates, 20 μ l were added in each
217 well, after 30 min of incubation the bioluminescence was evaluated on a plate reader
218 (SAFAS FLX-Xenius). After analysis of the protein content of the cell suspension
219 (Bradford method), the reducing capacity of the cells were expressed in arbitrary
220 units per μ g of protein/min.

221 **2.5 Statistical analyses**

222 Statistical analyses were performed with the Statistica® software. ANOVA and post
223 hoc tested were used when needed. When the data appeared not to follow the
224 conditions of normality (Shapiro-Wilk test) and of homoscedasticity (Levene test),
225 Wilcoxon tests were performed and eventually followed by Mann-Whitney tests. Data
226 were expressed as median \pm interquartile ranges (med \pm iq). Differences were
227 considered significant when $p < 0.05$.

228

229 **3. Results**

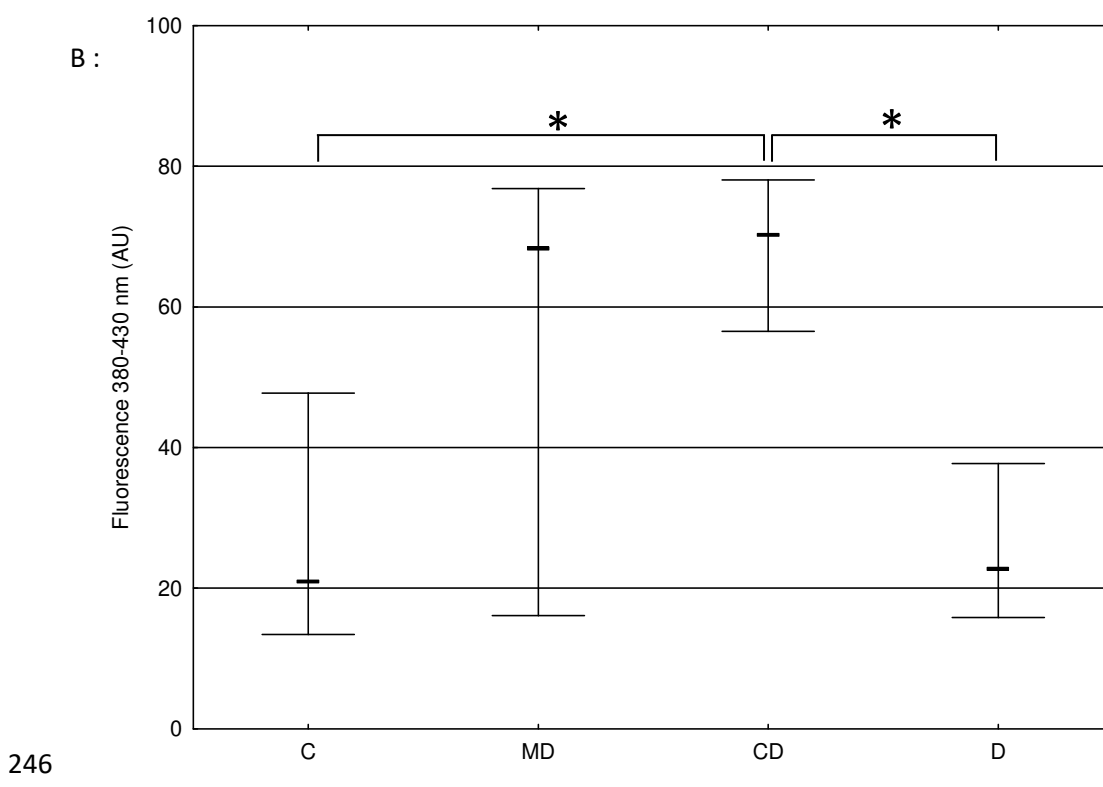
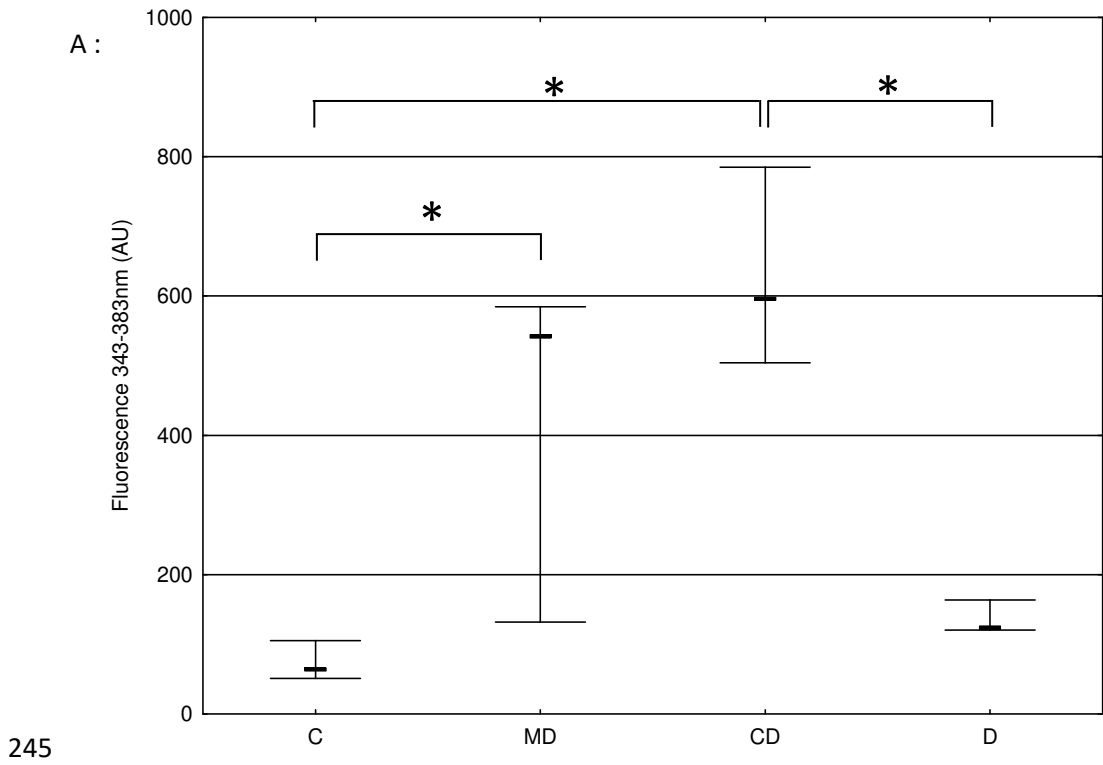
230 **3.1 Total Hydrocarbon concentration in seawater**

231 The total hydrocarbon concentration (THC) has been determined thanks to the
232 triplicate sampling performed every 12h during the 48h contamination period. In the
233 control and dispersant conditions THC was below the calibration limits (< to 0.5
234 mg/L). In the mechanical dispersion condition THC was $34,3 \pm 3.7$ mg/L and $43,6 \pm$
235 $4,5$ mg/L for the chemical dispersion of the oil.

236

237 **3.2 Biliary fluorescence**

238 Biliary fluorescence intensities at 380:430nm 343:383nm, after the 48h contamination
239 period are presented in **Figure 1**. In the MD group, 1-hydroxy-pyrene type
240 metabolites (343:383nm) were significantly increased when compared to the control
241 group. In the CD group both benzo[a]pyrene (380:430nm) and 1-hydroxy-pyrene
242 (343:383nm) type metabolites were increased when compared both to the control
243 and dispersant groups. In the dispersant group the biliary fluorescence was not
244 significantly modified when compared to the control group.



247 **Figure 1: Biliary fluorescence after the 48h exposure period.**

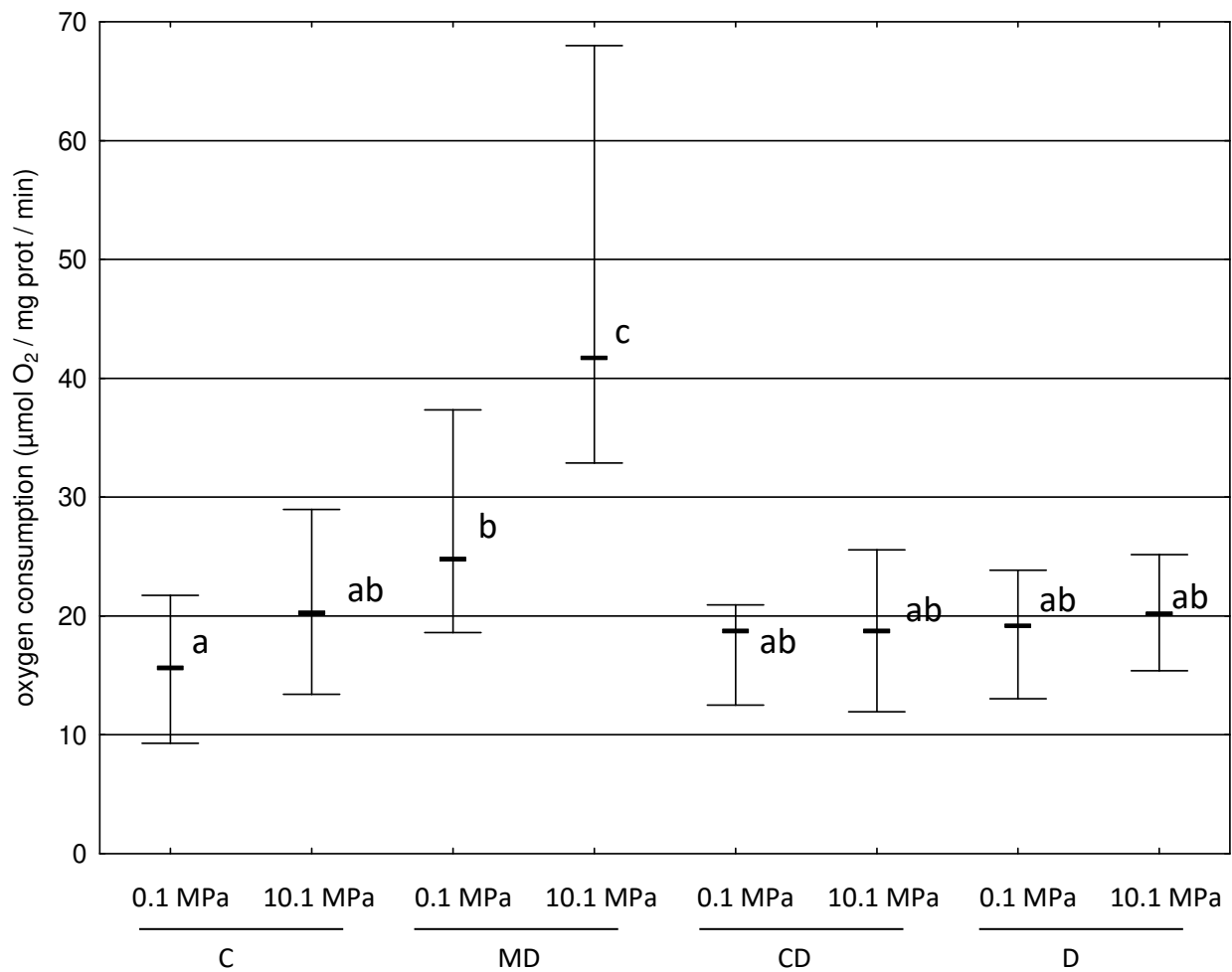
248 Fluorescence intensity at 343:383nm (A) and 380:430nm (B) in arbitrary units (AU).
249 C: control, MD: mechanical dispersion, CD: chemical dispersion, D: dispersant, *:
250 significant difference between group, $p < 0.05$, med \pm iq (n=6 to 7).

251

252 **3.3 Cellular oxygen consumption**

253 Hepatocyte oxygen consumptions were measured at atmospheric pressure and
254 under high hydrostatic pressure (see **Figure 2**). Exposure to 10.1 MPa induced a
255 significant increase of oxygen demand as well as contamination did (ANOVA
256 $p < 0,001$ and $p < 0,01$ respectively). In the control group, exposure to HP induced a
257 non-significant 30% increase of MO_2 . At 0.1 MPa, exposure to mechanically
258 dispersed oil induced a nearly 60% increase of MO_2 in comparison of control group,
259 and, when pressure was applied on hepatocytes, the MO_2 variation between C and
260 MD increased at 170%. Dispersant use (alone or in presence of oil) appeared to
261 prevent any increase of oxygen consumption either at atmospheric pressure or at
262 10.1 MPa.

263



264
265

266

267 **Figure 2: Oxygen consumption**

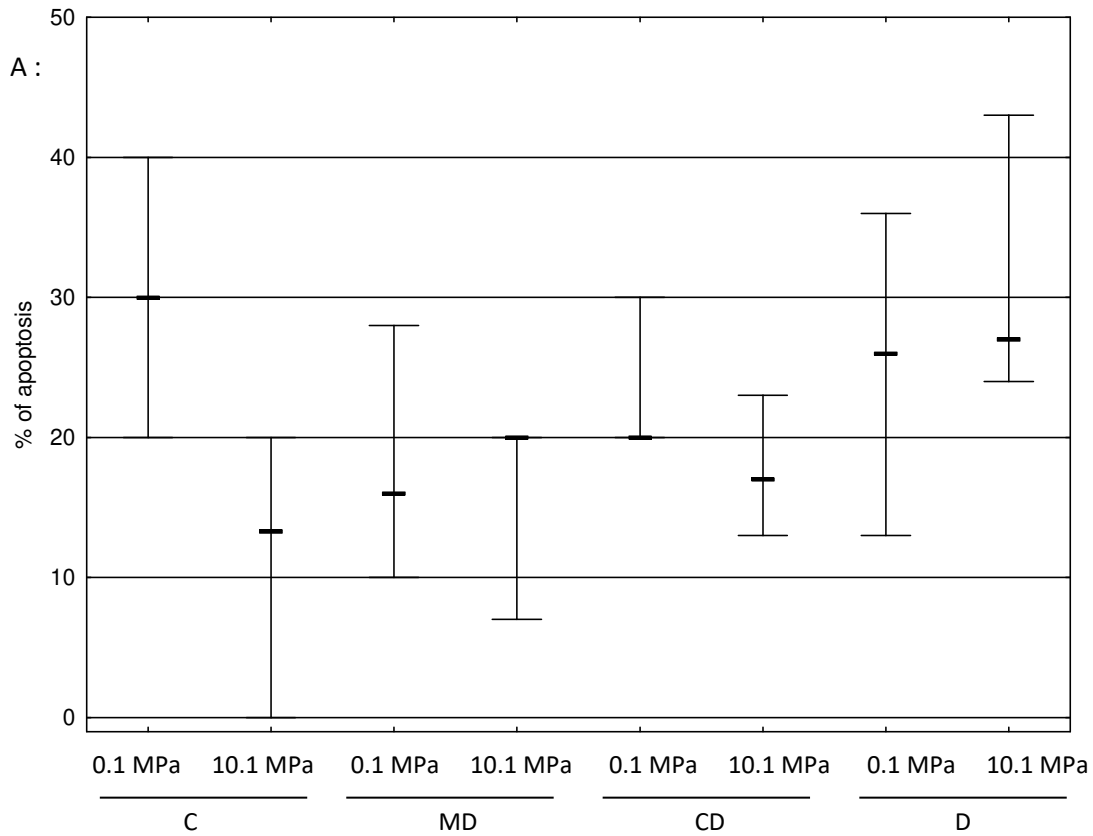
268 Hepatocyte oxygen consumption (in µmol O₂ / mg of protein / minute) for the different
 269 groups at atmospheric pressure (0.1 MPa) and under high hydrostatic pressure (10.1
 270 MPa) C: Control, MD: mechanical dispersion, CD: chemical dispersion D: dispersant.
 271 Letter differences indicate statistical differences. med +/- iq (n = 8 to 12).

272

273

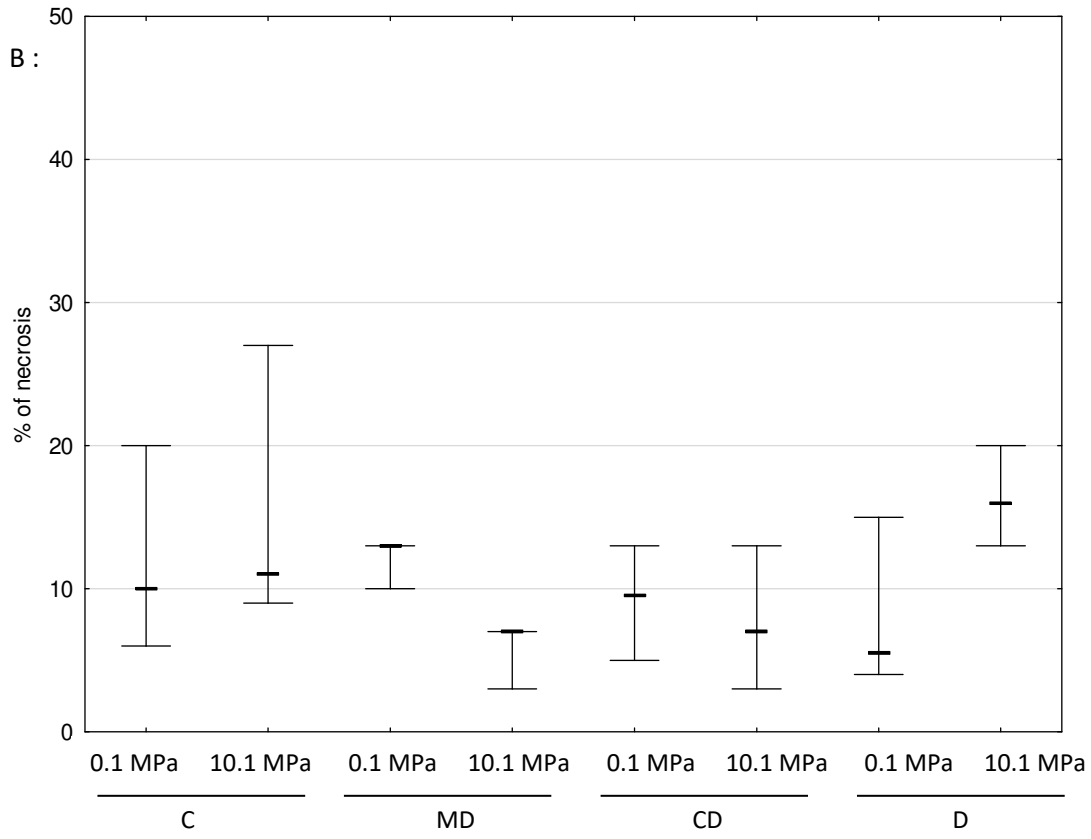
274 **3.4 Cell death, apoptosis & necrosis**

275 After hepatocytes staining with PI and Annexin V, cell mortality, apoptosis and
276 necrosis were calculated (**Figure 3**). In our experimental conditions, even if total
277 mortality seems to be slightly higher after exposure to the dispersant alone, none of
278 these parameters were significantly modified by contamination or hydrostatic
279 pressure exposure.



280

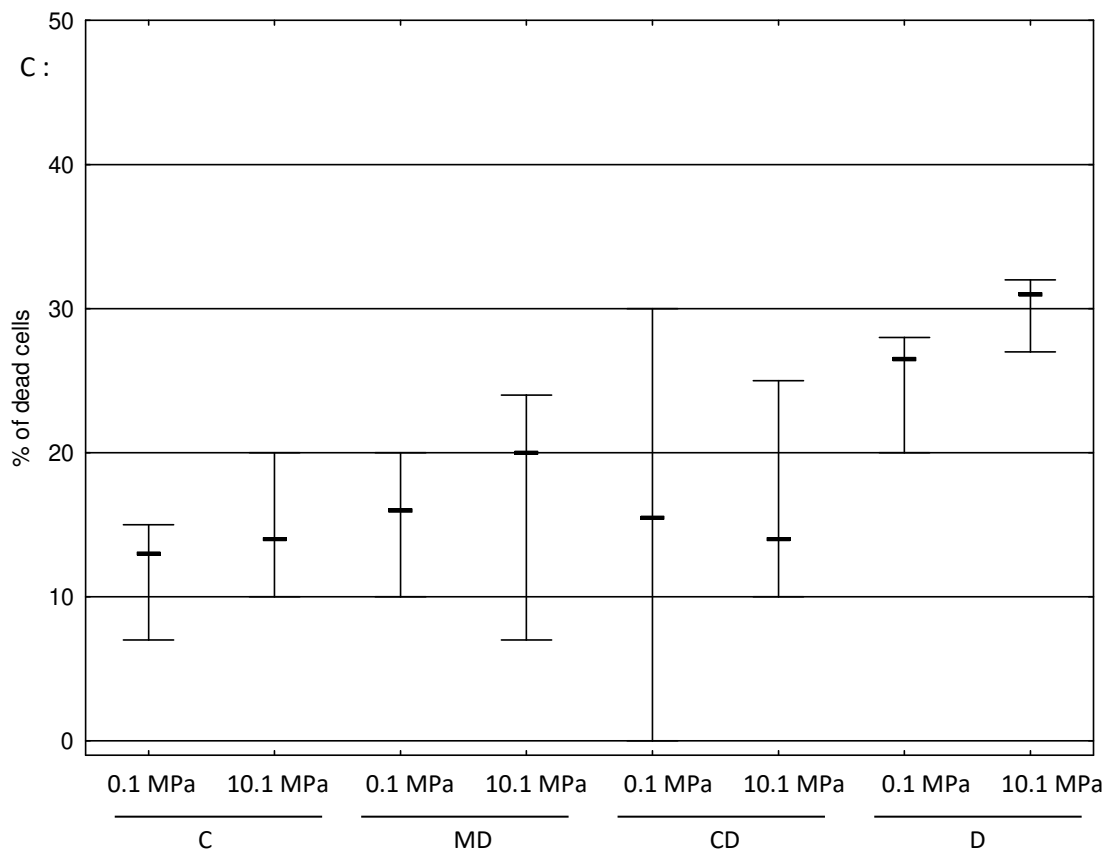
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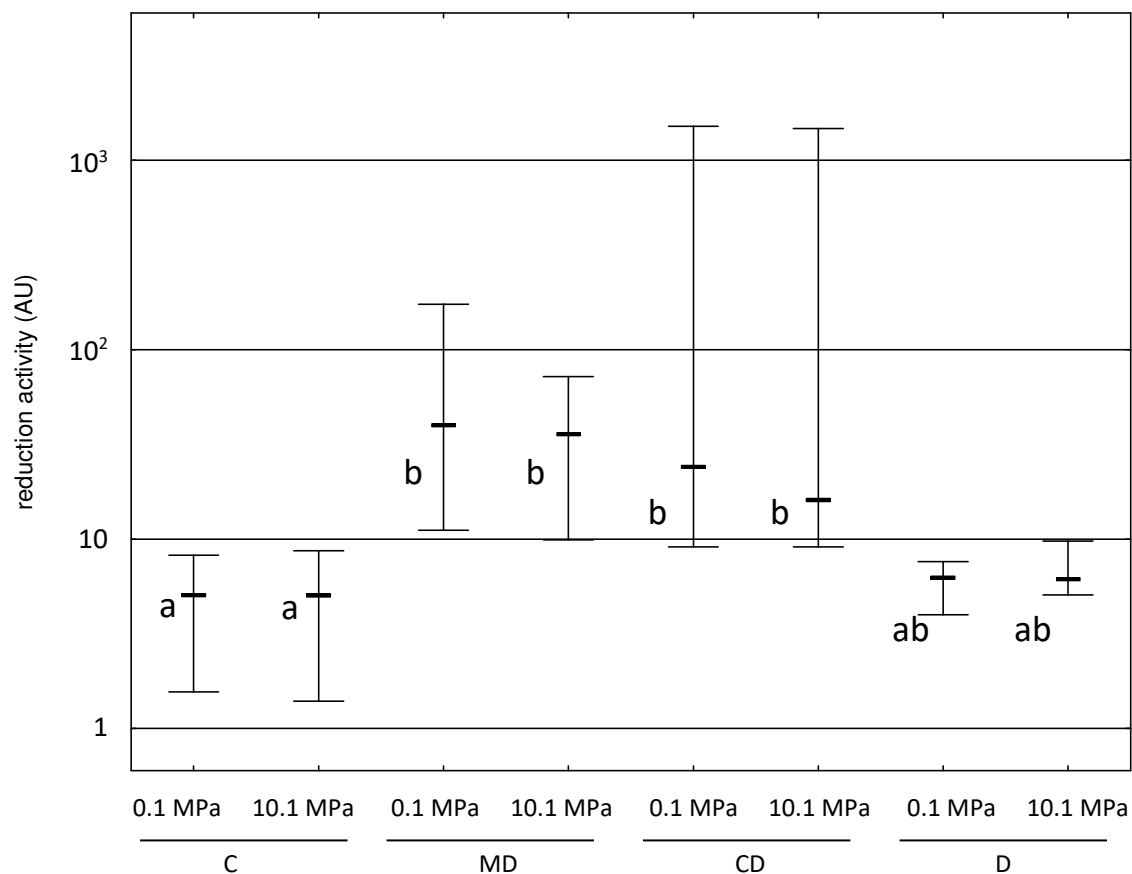
287 **Figure 3: Apoptosis, Necrosis and cell death**

288 Percentage of apoptosis (A), necrosis (B) and cell death (C) calculated from PI and
289 Annexin V staining for the different groups at atmospheric pressure (0.1 MPa) and
290 after exposure to high hydrostatic pressure (10.1 MPa). C: Control, MD: mechanical
291 dispersion, CD: chemical dispersion D: dispersant. No statistical differences between
292 groups could be demonstrated. med \pm iq (n = 10 to 12).

293

294 The reducing activity of the hepatocytes measured with the “Realtime-glo mt cell
295 viability” (Promega) were significantly increased by the oil exposure (either in MD or
296 CD groups) and was not modified by exposure to 10.1 MPa (**Figure 4**). The results
297 obtained in the case of the D group were intermediate and not significantly different
298 from C nor MD and CD groups.

299



300

301

302 **Figure 4: Hepatocyte reducing capacity**

303 Cell reduction capacity for the different groups at atmospheric pressure (0.1 MPa)
 304 and after exposure to high hydrostatic pressure (10.1 MPa). C: Control, MD:
 305 mechanical dispersion, CD: chemical dispersion D: dispersant. Letter differences
 306 indicate statistical differences. med +/- iq (n = 8 to 12).

307

308 **4. Discussion**

309 In the context of the development of the piezoecotoxicology, the goal of this study
 310 was to increase our understanding of the potential combined effects of oil/dispersant
 311 and hydrostatic pressure at a cellular level using turbot hepatocytes.

312 The choice of the turbot was based on several reasons. First, turbot is one of the
313 model fish for ecotoxicological test for OSPAR commission (OSPAR Protocols on
314 Methods for the Testing of Chemicals Used in the Offshore Oil Industry). But more
315 interestingly, turbot is present in coastal, estuarine and continental shelf areas from
316 the surface to at least 200 meters of depth and in all the European marine water from
317 Mediterranean Sea to Arctic circle (Imsland *et al.*, 2001). These various turbot
318 habitats demonstrate the strong adaptive capacities of this teleost species. The use
319 of a model species living in shallow and coastal but with good adaptive capacities
320 has been successfully used in other piezoecotoxicological studies. Firstly using
321 decapods, the shrimp, *Palaemon varians* was used in several studies performed at
322 different hydrostatic pressure and at lethal and sub-lethal cadmium concentrations
323 (Domingues, 2015). Secondly using fish, the seabass *Dicentrarchus labrax* has been
324 used during hyperbaric challenges coupled with oil exposure (Dussauze *et al.*, 2017).
325 Studies were also performed at cellular level: fish hepatocytes were exposed to 3-
326 methylcholanthrene at several hydrostatic pressure (Lemaire *et al.*, 2016).

327 The first steps of this study aimed to obtain turbot hepatocytes isolated from fish
328 exposed either to chemically dispersed oil, mechanically dispersed oil or dispersant
329 alone. The measurements of THC in the water and of the biliary fluorescence made it
330 possible to assess fish contamination. Neither control nor dispersant alone exhibited
331 oil contamination (THC in the water was below the detection limit and biliary
332 fluorescence were far below the exposed groups).

333 In this experimentation, the choice of the nominal concentration of dispersed oil
334 (80mg/L) was based on measurement made at sea after dispersant use on an oil spill
335 where THC can reach 50mg/L in the upper slice of the water column (Humphrey *et*

336 *al.*, 1987). For both MD and CD conditions, the THC values (respectively $34,3 \pm 3.7$
337 mg/L and $43,6 \pm 4,5$ mg/L) are lower than the nominal oil concentration (80mg/L) but
338 this difference can be explained by the oil adherence on the different surfaces of the
339 experimental system. Hence, our THC value even with a high nominal concentration
340 can be considered as realistic and more precisely in the upper range of oil
341 concentrations observed *in situ* during an oil spill.

342

343 The biliary fluorescence at 343-383 nm increases significantly in the MD group when
344 compared to control fish demonstrating the effectiveness of the contamination. In the
345 case of the chemical dispersion of the oil, the THC is $43,6 \pm 4,5$ mg/L and the biliary
346 fluorescence is increased at the two-wavelength couple analysed. Even if there is no
347 statistical difference between MD and CD groups, these results are compatible with
348 an increased bioavailability due to the dispersant use (Ramachandran *et al.*, 2004).

349

350 This study was designed to assess the effects of oil contamination on hepatocyte
351 oxygen consumption under high hydrostatic pressure. The analysis of cell death / cell
352 survival, apoptosis and necrosis were performed in order to be able to link an
353 eventual modification of MO_2 to cellular mortality.

354 The first point to consider, before analyzing the hepatocyte oxygen consumption, is
355 the strong divergence of results obtained with the two techniques used to evaluate
356 cell survival. The results from annexin V and PI staining show no change in
357 apoptosis, necrosis nor cell death in any of the conditions tested. In contrast, the
358 results of cell viability (determined with the "Realtime-glo mt cell viability" kit) show a
359 strong and significant increase in the MD and CD group independently of the

360 pressure exposure. In the case of this last kit, the cellular viability is indirectly
361 evaluated *via* the reducing activity of the hepatocytes; the measurement is based on
362 the reduction of a proprietary organic substrate by the cellular metabolism. The
363 subsequent increase of luminescence is classically interpreted as an index of cell
364 survival (see for example Hsieh *et al.*, 2017). In our case, the strong increase of
365 response in the presence of oil can hardly be analyzed as an increase of cell survival
366 since oil is clearly known to be hepatotoxic (Farmen *et al.*, 2010; Au DWT, 2004) and
367 since direct measurement of cell death (PI) and apoptosis (annexin V) were not
368 modified. We hypothesise that the observed modification of luminescence is due to
369 the enzymatic induction linked to the oil exposure of the fish. Polycyclic aromatic
370 hydrocarbon exposures are indeed known to induce (*via* the Aryl Hydrocarbon
371 receptor) the activation of a set of electron transport enzymes leading to their
372 biotransformation and metabolization (Gelboin, 1980; van der Oost *et al.*, 2003). The
373 induction of biotransformation enzymes *in vivo* during the contamination process
374 could led to an increased capacity of isolated hepatocytes to reduce the proprietary
375 molecule independently of any change in cell survival. The analysis of this hypothesis
376 could benefit from the knowledge of the proprietary organic substrate structure
377 (unfortunately unavailable). PI and annexin V staining being direct methods of cell
378 apoptosis and cell death, the discussion of hepatocyte oxygen consumption will be
379 done considering no statistical changes in cell survival between the tested conditions.

380

381 Hydrostatic pressure exposure is known to induce a strong increase of aerobic
382 metabolism in a number of species including fish and crabs and this increase is
383 classically interpreted as the result of an increase of motor activity (Sébert *et al.*,
384 1995). Hydrostatic pressure is also known to alter mitochondrial activity in trout (a

385 shallow–water species) but not in silver eel (a species facing HP during its life cycle)
386 (Sébert and Theron, 2001). As far as we know, the direct effect of HP on aerobic
387 metabolism of fish cells had never been measured. Our results show a significant
388 increase of the oxygen consumption in control hepatocytes exposed to HP (ANOVA
389 $p < 0,001$): an effect similar to the one observed at the level of the whole animal. Due
390 to the lack of literature on cell metabolism under HP, it is difficult to propose a
391 relevant hypothesis explaining the observed increase of the cellular energy demand.

392 Oil exposure at atmospheric pressure also induced an increase of the hepatocyte
393 MO_2 (ANOVA $p < 0.01$). It is likely that during the contamination process of fish, the
394 induction of hepatic biodegradation (van der Oost *et al.*, 2003) results in an increase
395 of the energetic cost at a cellular level and that this cost itself results in an increase of
396 the aerobic metabolism.

397 When HP is applied on hepatocytes from turbot previously exposed to the
398 mechanical dispersion of oil, the oxygen consumption sharply increases 2.7 times
399 from $15.6 \mu\text{molO}_2/\text{mg prot}/\text{min}$ (in the group control 0.1 MPa) to $41.8 \mu\text{molO}_2/\text{mg}$
400 prot/min (in the group MD 10.1 MPa). This result clearly evidences that oil
401 contamination and HP exposure have at least additive effects on the cellular
402 energetic demand. Interestingly dispersant use prevented any increase of oxygen
403 demand either after HP exposure and oil contamination. Previous experiments had
404 shown that exposure to dispersant induces an important reduction of the activity of
405 the mitochondrial respiratory chain in cardiac permeabilized muscle fibers in seabass
406 *Dicentrarchus labrax* (Tissier *et al.*, 2015) but not in polar cod *Boreogadus saida*
407 (Dussauze *et al.*, 2014). Based on the seabass result, and even if this study is
408 performed on hepatocytes, we could hypothesise that in the case of the turbot, the
409 dispersant prevents the increase of oxygen consumption by reducing the capacities

410 of energy production by the mitochondria and not by reducing the cellular energy
411 demand.

412 If this hypothesis is correct, it should have functional consequences at a higher
413 degree of integration. In particular, the ability of the turbot to cope with an acute HP
414 exposure should be reduced by an oil contamination in presence of dispersant
415 (because of a concomitant increased energy demand and reduced mitochondrial
416 capacity). Interestingly, this hypothesis is coherent with a previous study of Dussauze
417 *et al.* (2017) where we demonstrated reduced capacity of juvenile seabasses
418 exposed to dispersed fuel oil to face HP exposure.

419

420 **5. Conclusion**

421 The analysis of oxygen consumption from turbot hepatocytes at atmospheric
422 pressure and at 10.1 MPa after fuel oil contamination and/or chemical dispersant
423 demonstrates that fuel oil contamination and hydrostatic pressure have additive
424 effects on oxygen consumption at a cellular level and that dispersant prevents any
425 MO_2 increase. These results appear to be in good coherence with the reduced
426 capacity of juvenile turbot to face HP exposure after exposure to a chemically
427 dispersed fuel oil. The mechanisms leading to this increased energetic demand and
428 its eventual inhibition will have to be investigated in further experiments.

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432 **References**

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