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1 **Liver antioxidant and plasmatic immune responses in juvenile**
2 **golden grey mullet (*Liza aurata*) exposed to dispersed crude oil**

3
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26
27 **Abbreviations:** BAL : Brut Arabian Light ; C : Control ; CD : Chemically
Dispersed oil ; D : Dispersant solution ; MD : Mechanically Dispersed oil; WSF :
Water Soluble Fraction

28 **Abstract**

29
30

31 **Dispersant application is an oil spill response technique. To evaluate the environmental**
32 **cost of this operation in nearshore habitats, the experimental approach conducted in this**
33 **study exposed juvenile golden grey mullets (*Liza aurata*) for 48 hours to chemically**
34 **dispersed oil (simulating, *in vivo*, dispersant application), to dispersant alone in sea**
35 **water (as an internal control of chemically dispersed oil), to mechanically dispersed oil**
36 **(simulating, *in vivo*, natural dispersion), to the water-soluble fraction of oil (simulating,**
37 ***in vivo*, an oil slick confinement response technique) and to sea water alone (control**
38 **condition). Biomarkers such as fluorescence of biliary polycyclic aromatic hydrocarbon**
39 **(PAH) metabolites, total glutathione liver content, EROD (7-ethoxy-resorufin-O-**
40 **deethylase) activity, liver antioxidant enzyme activity, liver lipid peroxidation and an**
41 **innate immune parameter (haemolytic activity of the alternative complement pathway)**
42 **were measured to assess the toxicity of dispersant application. Significant responses of**
43 **PAH metabolites and total glutathione liver content to chemically dispersed oil were**
44 **found, when compared to water-soluble fraction of oil. As it was suggested in other**
45 **studies, these results highlight that priority must be given to oil slick confinement**
46 **instead of dispersant application. However, since the same patterns of biomarkers**
47 **responses were observed for both chemically and mechanically dispersed oil, the results**
48 **also suggest that dispersant application is no more toxic than the natural dispersion**
49 **occurring in nearshore areas (e.g. waves). The results of this study must, nevertheless, be**
50 **interpreted cautiously since other components of nearshore habitats must be considered**
51 **to establish a framework for dispersant use in nearshore areas.**

52

53 **Keywords:** dispersed crude oil; dispersant; oxidative stress; complement system; *Liza aurata*;
54 nearshore areas.

55 **1. Introduction**

56

57 By accelerating the dispersion of oil from the sea surface into the water column, the use of
58 dispersants (surface active agents) offers the environmental benefits of (i) diluting the oil slick
59 in the water column (Lessard and DeMarco, 2000), (ii) reducing the threat of oiling shorelines
60 and (iii) accelerating the bacterial degradation of oil by increasing the available surface of the
61 oil (Tiehm, 1994; Churchill et al., 1995). However, the use of dispersant is, at the moment,
62 subject to certain restrictions depending mainly on weather conditions, oil type, distance to
63 the shore and/or water depth. For example in European Atlantic coast the minimum permitted
64 water depth is 10 m (Chapman et al., 2007). This restriction of minimum water depths was
65 derived from studies on the dilution of dispersed oil in shallow water and took into
66 consideration the ecological sensitivity of nearshore areas as they are nurseries for many
67 aquatic species. However, a field study conducted by Baca et al. (2005) suggests that, in
68 nearshore tropical ecosystems, dispersant use minimizes the environmental damages arising
69 from an oil spill. This Net Environmental Benefits Analysis (NEBA) highlights a positive
70 environmental role of dispersant use in nearshore areas but it is only applicable to tropical
71 mangroves. To the best of our knowledge no NEBA has ever been conducted in Atlantic
72 coastal ecosystems in order to establish the current restrictions for dispersant use and policies
73 in nearshore areas. To do so, an on-going project (DISCOBIOL project: DISpersant and
74 response techniques for COastal areas; BIOLogical assessment and contributions to the
75 regulation) aims at obtaining informations on the environmental impact of dispersed oil in
76 nearshore areas.

77 Including in this project, this study aims at assessing the toxicity of chemically dispersed oil
78 at concentration similar to those encountered at oil spill sites. To simulate current operational
79 oil dispersant application, our study uses a third generation dispersant, which is the more

80 recent formulations and is considered as the less toxic, the more concentrated in tensio-active
81 and there by the most commonly used at the moment. While, most experimental studies
82 assessed the toxicity of the dispersant itself (Adams et al., 1999; George-Ares and Clark,
83 2000) or the dispersed oil water-accommodated fraction (Cohen and Nugegoda, 2000;
84 Mitchell and Holdway, 2000; Ramachandran et al., 2004; Perkins et al., 2005; Jung et al.,
85 2009), our experimental approach simulates operational oil dispersant application,
86 considering the presence of oil droplets in the water column. Indeed, oil droplets are
87 suggested to be a determinant of toxicity (Brannon et al., 2006) and does so even more in
88 nearshore areas, where natural dispersion (e.g. waves) can replace the whole oil slick from the
89 surface in the water column (as described during the Braer oil spill by Lunel, 1995).

90 To reveal the toxicity of this chemically dispersed oil, several biomarkers were assessed after
91 exposure of juvenile golden grey mullets (*Liza aurata*). The choice of the species is due to (i)
92 its presence in nearshore areas during its early life stages (Gautier and Hussenot, 2005) and
93 consequently its status of pollutants target organism (Bruslé, 1981); and to (ii) its significant
94 role in the coastal ecosystems, since this fish species permits an important particulate organic
95 matter transport from the salt marsh to the marine coastal waters (Lafaille et al., 1998).

96 In this context, the use of biomarkers seems appropriate since they are defined as “a
97 biochemical, cellular, physiological or behavioural variation that can be measured in tissue or
98 body fluid samples or at the level of whole organisms that provides evidence of exposure to
99 and/or effects of one or more chemical pollutants” (Depledge et al., 1995). Hence, these
100 ecotoxicological tools provide integrative informations, linking exposure to pollutants and the
101 health of the monitored organisms (Sanchez and Porcher, 2009). As a consequence, other
102 studies evaluate the toxicity to fish of a dispersed crude oil through biomarkers assessment
103 (Cohen and Nugegoda, 2000; Jung et al., 2009; Mendonça Duarte et al., 2010) and reveal an
104 increase of toxicity due to dispersant application. In our study, a set of complementary

105 biomarkers, including EROD (7-ethoxy-resorufin-O-deethylase) activity implicated in phase I
106 biotransformation, total glutathione (GSH), enzymatic antioxidant activities (glutathione
107 peroxidase, GPx; catalase, CAT; superoxide dismutase, SOD; glutathione-S-transferase, GST)
108 and lipid peroxidation (LPO) were measured in the liver of golden grey mullet. These
109 biomarkers are known to be sensitive to petroleum compounds and in particular to polycyclic
110 aromatic hydrocarbons (PAHs) as described in Pan et al. (2005), Oliveira et al. (2008),
111 Nahrgang et al. (2009) and Hannam et al. (2010). Moreover, the physiological links between
112 the presence of PAHs, the production of radical oxygen species (ROS) and consequently
113 enzymatic and non-enzymatic antioxidant responses have also been described (Stegeman,
114 1987; Livingstone, 2001). The haemolytic activity of the alternative complement pathway
115 (ACH 50), an innate immune parameter that is involved in the innate humoral response, was
116 measured in the plasma of the golden grey mullets, since it is a known biomarker of
117 petroleum exposure (Bado-Nilles et al., 2009). Modulations of the antioxidant system and
118 innate immune function will be discussed with regards to the 16 PAHs USEPA priority
119 pollutants, the concentration of total petroleum hydrocarbons (TPHs) in seawater and
120 exposure biomarkers: pyrene-derived and benzo[*a*]pyrene-derived biliary metabolites.

121

122 **2. Materials and methods**

123

124 2.1. Chemicals

125

126 An Arabian Crude Oil containing 54% saturated hydrocarbons, 36% aromatic hydrocarbons
127 and 10% polar compounds, was selected for this study. Before exposure, the oil was
128 evaporated (in a 1m³ tank, during 24 hours) under atmospheric conditions and natural UV-
129 sunlight in order to simulate the natural behaviour of the oil after it is released at sea

130 (evaporation of light compounds and natural photodegradation, respectively). The resulting
131 chemical composition of the oil was 54% saturated hydrocarbons, 34% aromatic
132 hydrocarbons and 12% polar compounds.

133 With regards to dispersant, a formulation manufactured by Total Fluides was selected based
134 on its efficiency. Dispersant was evaluated by CEDRE (Centre de Documentation de
135 Recherche et d'Expérimentations sur les pollutions accidentelles des eaux, France) and was
136 deemed effective enough to be used in the marine environment (preliminary determined using
137 the method NF.T.90-345), non-toxic at the concentration recommended by the manufacturer
138 (preliminary determined assessing standard toxicity test: method NF.T.90-349) and
139 biodegradable. Its chemical formulation was not available for reasons of confidentiality.

140

141 2.2. Experimental animals

142

143 The experiment was carried out using 50 juvenile golden grey mullets (*Liza aurata*), which
144 were provided by Commercio Pesca Novellame Srl, Chioggia, Italy. Their average length was
145 139.0 ± 0.7 mm (mean \pm standard error of the mean) and their average weight was $38.25 \pm$
146 1.22 g.

147 The fish were acclimatized for 3 weeks in 300-L flow-through tanks (dissolved oxygen: $91 \pm$
148 2%; salinity: $35 \pm 1\%$; 15 ± 0.1 °C, with a 12 h light:12 h dark photoperiod in seawater free of
149 nitrate and nitrite) prior to the exposure studies. During acclimation, they were fed daily with
150 fish food (Neosupra AL3 from Le Gouessant aquaculture) but were starved for 48 h prior to
151 the bioassays and throughout the exposure period, in order to avoid bile evacuation from the
152 gallbladder.

153

154 2.3. Experimental design

155

156 2.3.1. Experimental system

157

158 The experimental system (**Figure 1**) was devised to maintain the mixture of oil and dispersant
159 as a homogenous solution. The mixture was homogenized using a funnel (at the surface of a
160 300-L seawater tank), which was linked to a Johnson L450 water pump (at the bottom of the
161 tank) in order to homogenize the mixture despite the hydrophobic nature of the oil.
162 Preliminary tests showed that, after 24 hours of homogenisation, the total petroleum
163 hydrocarbon (TPH) concentrations in the water column do not depend on water column depth,
164 suggesting the homogenous dispersion of small petroleum droplets throughout the water
165 column. The system was a static water system stocked in a temperature controlled room (15
166 °C), and thus exposure studies were conducted at 15 ± 0.1 °C. Other physico-chemical
167 parameters were also measured: pH (8.02 ± 0.07) and dissolved oxygen ($95 \pm 1\%$) remained
168 constant throughout the study.

169

170

171 2.3.2. Exposure conditions and exposure media

172

173 Control exposure medium (C) was made up using seawater provided by Oceanopolis, Brest,
174 France. The chemically dispersed (CD) oil exposure medium was made by pouring 20 g of
175 petroleum and 1 g of dispersant into the funnel of the experimental system. Dispersant alone
176 (D) exposure medium, as an internal control of CD, was made by pouring 1 g of dispersant
177 into the funnel. The mechanically dispersed (MD) oil exposure medium was made by pouring
178 20 g of petroleum into this funnel. For the water-soluble fraction of oil (WSF), in addition to
179 the funnel and the pump which were kept to maintain the same level of agitation of the

180 seawater as for other treatments, a 20 g oil slick was contained using a plastic cylinder (21 cm
181 diameter) placed on the surface of the seawater (4 cm below the surface and 8 cm above). A
182 plastic mesh was placed at the bottom of the plastic cylinder. The spreading of the oil slick
183 was not prevented by the plastic cylinder, as the oil slick was smaller in diameter than the
184 plastic cylinder, therefore the experimental approach simulates the actual spreading behaviour
185 of oil at sea. During the entire exposure period, the oil slick remained at the surface without
186 mixing and the fish were only exposed to the soluble fraction of the oil.

187 None of the fish were exposed for 24 hours, while the solutions remained homogenous. The
188 groups of 5 fish were then randomly distributed in the five experimental tanks, each tank
189 containing an exposure medium (described above). The fish were exposed to the different
190 media for a period of 48 h and the protocol was replicated so that 10 fish were exposed to
191 each exposure medium.

192 At the end of the exposure period, the fish in each tank (each exposure medium) were
193 euthanized using eugenol (4-allyl-2-methoxyphenol). To collect plasma samples, 0.2 mL of
194 blood was withdrawn from the caudal vein of each fish and centrifuged (12,000 g, 10 min, 4
195 °C, Jouan). Plasma samples were stored at -80 °C. The liver and gallbladder were removed
196 from each fish and stored at -80 °C prior to analysis.

197

198

199 2.4. TPH and PAH concentrations

200

201 2.4.1. TPH seawater concentrations

202

203 The TPH concentration, which is the sum of dissolved hydrocarbon concentrations plus the
204 amount of oil droplets, was measured for all exposure media at the beginning (T=0 h) and at

205 the end of fish exposure (T=48 h), using the mean of three replicated measurements for each
206 time point. The seawater samples were extracted with 10 mL of pestipur-quality
207 dichloromethane (99.8 % pure solvent, Carlo Erba Reactifs, SDS). After separation of the
208 organic and aqueous phases, water was extracted two additional times with the same volume
209 of dichloromethane (2 x 10 mL). The combined extracts were dried on anhydrous sulphate
210 and then analyzed using a UV spectrophotometer (UV-Vis spectrophotometer, Unicam) at 390
211 nm, as described by Fusey and Oudot (1976).

212

213 2.4.2. Seawater concentrations of PAHs

214

215 PAH concentrations were assessed at the beginning (T=0 h) and at the end of fish exposure
216 (T=48 h), using the mean of three replicated measurements for each time point. After
217 sampling, the first step was a 24-hour settling phase to separate oil droplets and particulate
218 matter from the seawater. Then, PAHs were extracted from the seawater using the stir bar
219 sorptive extraction technique (SBSE – Stir bar coated with PDMS, Gerstel), and analyzed
220 using thermal desorption coupled to capillary gas chromatography-mass spectrometry (GC–
221 MS). The GC was a HP7890 series II (Hewlett Packard, Palo Alto, CA, USA) coupled with a
222 HP5979 mass selective detector (MSD, Electronic Impact: 70eV, voltage: 2 000 V). PAHs
223 were quantified according to published procedures (Roy et al., 2005).

224

225 2.5. Biochemical analyses

226

227 2.5.1 Fixed wavelength fluorescence analysis

228

229 Bile samples were diluted (1:250) in absolute ethanol (VWR International). Fixed wavelength
230 fluorescence (FF) was then measured at the excitation:emission wavelength pairs 341:383 and

231 380:430 nm. FF 341:383 mainly detects pyrene-derived metabolites and FF 380:430 mainly
232 detects benzo[*a*]pyrene-derived metabolites (Aas et al., 2000). Measurements were performed
233 in quartz cuvettes on a spectrofluorimeter (SAFAS Flx-Xenius). The FF values were
234 expressed as arbitrary units of fluorescence and the signal levels of pure ethanol were
235 subtracted.

236

237 2.5.2 Measurement of oxidative stress biomarkers

238

239 Livers were homogenized in ice-cold phosphate buffer (100 mM, pH 7.8) containing 20%
240 glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a serine protease inhibitor. The
241 homogenates were centrifuged at 10,000 g, 4 °C, for 15 min and the postmitochondrial
242 fractions were used for biochemical assays. Total protein concentrations were determined
243 using the method of (Bradford, 1976) with bovine serum albumin (Sigma-Aldrich Chemicals,
244 France) as a standard. Hepatic biomarkers assays including GSH content and activities of
245 EROD, GST, GPx, SOD and CAT were adapted for use in microplate and, after preliminary
246 test using several dilutions, adapted for samples of liver of juvenile golden grey mullet.

247 The EROD activity was measured using the fluorimetric assay developed by Flammarion et
248 al. (1998). To summarize, 10 µL of a 5g proteins/L diluted sample were added to phosphate
249 buffer containing 8 µM of 7-ethoxyresorufin and 0.5 mM of NADPH. Formed resorufin was
250 quantified by fluorimetric measurement with 530 nm wavelength excitation and 590 nm
251 wavelength emission. Resorufin was used as standard, and results were expressed as nmol
252 resorufin/min/g protein.

253 The GSH (total glutathione) concentration was measured according to Vandeputte et al.
254 (1994). Briefly, 10 µL of TCA-deproteinized sample were mixed with phosphate buffer
255 containing 0.3 mM NADPH and 1 mM Ellman reagent. The enzymatic reaction was

256 monitored spectrophotometrically at 405 nm and the results were expressed in μmol of GSH/g
257 of proteins.

258 The GST activity assay was conducted according to Habig et al. (1974). Briefly, 10 μL of a
259 0.75 g proteins/L diluted sample were mixed with 1 mM chloro dinitro benzene and 1 mM
260 reduced glutathione. The enzymatic reaction was monitored spectrophotometrically at 340 nm
261 and the results were expressed in U of GST/g of proteins.

262 GPx activity was determined using 15 μL of a 4.5 g proteins/L diluted sample according to
263 the method of Paglia and Valentine, (1967). Cumene hydroperoxide was used as the substrate
264 and enzymatic activity was assessed at 340 nm. The results were expressed in U of GPx/g of
265 proteins.

266 SOD activity was measured using the assay developed by Paoletti et al. (1986). Briefly, the
267 inhibition of NADH (350 μM) oxidation by 20 μL of a 0.25 g proteins/L diluted sample was
268 monitored at 340 nm. The results were presented in U of SOD/mg of proteins.

269 CAT activity was monitored using the method previously described by Babo and Vasseur
270 (1992). Briefly, 0.08 g proteins/L diluted samples were mixed (v:v) with 28 mM hydrogen
271 peroxide. The kinetics of hydrogen peroxide degradation were assessed at 280 nm and the
272 results were expressed in U of CAT/mg of proteins.

273

274 2.5.3 Lipid peroxidation (LPO) determination

275

276 Lipid peroxidation levels were assessed via malondialdehyde (MDA) content determined
277 using a commercially available MDA assay kit (Oxis International MDA assay kit). The
278 method was based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with
279 MDA at 45 °C. The blue product was quantified by measuring absorbance at 586 nm (Gérard-
280 Monnier et al., 1998).

281

282 2.5.4 Determination of the alternative pathway of plasma complement activity

283

284 Determination of the alternative pathway of plasma complement activity was carried out by
285 haemolytic assay with rabbit red blood cells (RRC, Biomérieux, France) as described by
286 Yano (1992) and adapted to microtitration plates. Plasma samples, diluted to 1/80 in EGTA-
287 Mg-GVB buffer, were added in increasing amounts, from 10 to 100 μL per well. The wells
288 were then filled with EGTA-Mg-GVB buffer to a final volume of 100 μL . Finally, 50 μL of a
289 suspension containing 2% rabbit red blood cells were added to each well. Control values of
290 0% and 100% haemolysis were obtained using 100 μL of EGTA-Mg-GVB buffer and 100 μL
291 of non-decomplemented trout haemolytic serum at 1/50 in ultra pure water respectively.
292 Samples were incubated for 1 hour at 20 °C. The microplates were centrifuged (400 g, 5 min,
293 4 °C, Jouan). Then, 75 μL of supernatant from each well were transferred with 75 μL of
294 phosphate buffer saline (Biomérieux, France) into another 96-well microplate. The
295 absorbance (540 nm) was read in a spectrofluorimeter (SAFAS Flx-Xenius) and the number
296 of ACH 50 units per mL of plasma was determined by reference to 50% haemolysis.

297

298 2.6 Statistical analysis

299

300 The statistical analysis was carried out using XLstat 2007 software. The assumptions of
301 normality and homoscedasticity were verified using the Kolmogorov-Smirnov and Cochran
302 tests, respectively. Firstly, Student's t-tests were conducted, for each variables (fixed
303 wavelength fluorescence, EROD activity, total glutathione concentration, hepatic oxidative
304 stress biomarkers, lipid peroxidation, haemolytic activity of alternative complement pathway)
305 in order to highlight significant differences between both experimental replicates of each

306 exposure media. No significant difference was found, thereby, both replicates were
307 considered as one homogenous group of ten individuals. A factorial analysis of variance (one-
308 way ANOVA) was performed in order to assess the effects of the several exposure conditions.
309 This statistical analysis was followed by the Tukey post-hoc test to detect significant
310 differences between groups. The significance of the results was ascertained at $\alpha=0.05$. The
311 results were expressed as means \pm s.e.m. (standard error of the mean) corresponding to groups
312 of ten fish (n=10).

313

314

315 **3. Results**

316

317 No fish mortality was observed during the experiments. Moreover no TPH or PAH was
318 detected in the control and dispersant exposure media. The TPH concentration measured in
319 the CD (chemically dispersed oil) and MD (mechanically dispersed oil) groups corresponded
320 to that encountered under oil spill situations (for instance, 1 to 100 mg/L of total petroleum
321 hydrocarbons were measured in coastal waters around Shetland during the Braer oil spill, as
322 reported by Lunel,1995). No oil slick was observed in either the CD or MD exposure media,
323 suggesting that the energy in the experimental system was sufficient to disperse the oil slick.
324 These observations validated the experimental procedure.

325

326 3.1. Total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAH)
327 concentration in seawater.

328

329 The TPH concentrations of oil were higher in media with dispersant compared to without, and
330 the lowest concentration was observed in the WSF (water soluble fraction of oil) medium, in

331 which only dissolved compounds were present in the seawater column. In the CD medium,
332 the TPH concentration (**Table 1**) was 39 mg/L at the beginning of the exposure period (T=0
333 h) and 25 mg/L at the end of the exposure period (T=48 h), giving a percentage decrease of 36
334 %. In the MD medium, the TPH concentration was 13 mg/L at the beginning of the exposure
335 period (T=0 h) and 9 mg/L at the end of the exposure period (T=48 h), giving a percentage
336 decrease of 29 %. The TPH concentration could not be determined in the WSF medium since
337 it was too low to be detected using spectrophotometry.

338 According to spectrophotometry as well as gas chromatography coupled with mass
339 spectrometry, petroleum compounds and PAHs were not detected in the D (Dispersant) or C
340 (Control) media.

341 In terms of the sum of 16 parent and alkylated USEPA PAHs (Σ PAH) concentrations, the CD
342 medium contained 43.98 $\mu\text{g/L}$, at the beginning of the experiment, then 26.34 $\mu\text{g/L}$ after 48
343 hours, giving a percentage decrease of 40 %. For MD, the percentage decrease was 48 %: the
344 Σ PAH concentration at T=0 h was 39.09 $\mu\text{g/L}$ and at T=48 h it was 20.63 $\mu\text{g/L}$. WSF values
345 were lower when compared to both the CD and MD values, with a Σ PAHs concentration at
346 T=0 h of 5.16 $\mu\text{g/L}$ and at T=48 h of 0.47 $\mu\text{g/L}$, corresponding to a drastic decrease (91 %).

347 Regarding the concentration of 16 USEPA PAHs (alkylated and parents) in seawater during
348 CD, MD and WSF exposures (**Table 2**), it appears that two- or three-ring PAH compounds
349 (specifically, naphthalene alkylated compounds) were dominant when compared to heavier
350 PAHs (\geq four rings). Regarding the variation over time in PAH concentration, it appears that
351 light PAHs such as naphthalene (parent and alkylated) decreased during CD, MD and WSF
352 exposure (with the exception of fluorene for CD exposure) while the concentrations of
353 heavier PAHs remained relatively stable or increased (e.g. chrysene).

354

355 3.2. Fixed wavelength fluorescence analysis of biliary PAH metabolites

356

357 With regards to the levels of benzo[*a*]pyrene-type metabolites, which were measured by
358 fluorescence intensity (FF 380:430), CD and MD exposures led to significantly higher values,
359 compared to the values obtained in control fish (C). The intensity of fluorescence did not
360 significantly differ between the C, WSF and D groups of fish, even though WSF exposure
361 seemed to increase the intensity (**Figure 2a**).

362 With regards to the levels of pyrene-type metabolites (**Figure 2b**), which were measured by
363 fluorescence intensity (FF 341:383), CD and MD exposure led to significantly higher values
364 when compared to values obtained in control fish (C). The intensity of fluorescence did not
365 significantly differ between the C, WSF and D groups of fish, even though WSF exposure
366 seemed to increase the intensity. The intensity of fluorescence following CD exposure was
367 significantly different to that following WSF and D exposure while it appears that MD
368 exposure did not induce an increase in fluorescence compared to WSF and D exposure.

369

370 3.3. EROD (7-ethoxy-resorufin-O-deethylase) activity, Total glutathione content and hepatic
371 oxidative stress biomarkers

372

373 EROD demonstrated no significant difference between the exposure conditions (**Figure 3**)
374 and was characterized by a high intragroup variability that could reflect differences in
375 biotransformation processes between organisms.

376 The concentration of GSH (total glutathione, **Figure 4**) significantly decreased after exposure
377 to CD (45.35 ± 8.65) and MD (53.18 ± 10.04), compared to the control group ($130.50 \pm$
378 32.64), while no significant difference was observed after exposure to WSF (90.51 ± 23.11)
379 or D (108.44 ± 22.86). When CD and MD were compared with WSF and D, no significant

380 difference was revealed even though the GSH content in the WSF and D groups seemed
381 higher than in the CD and MD groups.

382 No significant difference between exposure conditions was recorded for antioxidant enzyme
383 activities (i.e. GST, GPx, SOD and CAT) (**Figure 5**).

384

385 3.4. Lipid peroxidation (LPO)

386

387 As for antioxidant enzymes, LPO demonstrated no significant difference between the
388 exposure conditions (**Figure 6**). However, LPO was characterized by a high intragroup
389 variability (especially for WSF, CD and MD exposure media) that could reflect differences in
390 sensitivity between organisms.

391

392 3.5. Haemolytic activity of alternative complement pathway (ACH 50)

393

394 The results are presented in **Figure 7**. As for antioxidant enzymes, ACH 50 demonstrated no
395 significant difference between the exposure conditions. Haemolytic activity appeared to be
396 lower after CD exposure and higher after MD exposure.

397

398 **4. Discussion**

399

400 The aim of this study was to accurately simulate operational oil dispersant application and to
401 assess its toxicity. An experimental system providing mixing energy (described in section
402 2.3.1) was necessary for this purpose: to achieve the dispersion of crude oil through
403 operational dispersant application, seawater energy is necessary (Merlin, 2005). Readers must
404 take into account that the results obtained (and discussed below), through this experimental

405 approach, are available only for a given mixing energy (the mixing energy induces by the
406 waterpump). However, extrapolation of results from the experimental approach to the oil spill
407 operations is possible. Indeed, meteorological conditions during the Braer oil spill (Wind
408 force 7 to 10, Lunel, 1995) were the most propitious to dispersed oil, among most of the
409 meteorological conditions during oil spills. While a dispersion of the whole oil was
410 maintained for more than one week, other oil spills, in offshore areas, exposed an unstable
411 dispersion of oil slick with a rapid decrease of concentration in 2-5 hours (Lessard and
412 Demarco, 2000). Our experimental approach is situated between these two opposite scenarios
413 (decrease of concentration on a 48 hours period, discussed in 4.1) and thus can be considered
414 as a possible one. Moreover, according to CEDRE observations during oil spill response in
415 nearshore area, 4 tide cycles (48h) are sufficient to totally disperse the oil slick, so that no
416 petroleum is present after this period. This suggests that an exposure of 48 h seems to be
417 accurate.

418 The fish were exposed to (i) a chemically dispersed oil (simulating dispersant application), (ii)
419 dispersant alone in sea water (as an internal control of chemically dispersed oil), (iii)
420 mechanically dispersed oil (simulating natural dispersion), (iv) water-soluble fraction of oil
421 (simulating an oil slick confinement response technique) and to (v) sea water alone (control
422 condition).

423 .

424 4.1. Total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAH)
425 concentrations in seawater.

426

427 The energy supplied by the experimental system was the same for the five exposure media.
428 However, our results show that the TPH concentration in the water column was higher in CD
429 than in MD at T=0 h and T=48 h. This finding and our observations, suggest that oil adheres

430 more to the experimental system in the MD exposure medium than in the CD exposure
431 medium. When extrapolated to field operations in the shallow water of nearshore areas, the
432 results show that the application of dispersants would promote higher concentrations of TPH
433 in the water column but would decrease the adherence to substrates (seagrass beds, sediments
434 etc...). This result is in accordance with Baca et al. (2005) and shows that dispersant
435 application increases the exposure to TPH for pelagic organisms living in the water column
436 (as golden grey mullets), while decreases the exposure to TPH for benthic organisms.

437 Unlikely TPH concentration, the difference of the sum of PAH concentrations between CD
438 and MD exposures is low (slightly higher in CD exposure). The sum of PAH concentration is
439 relevantly lower in WSF exposure medium than in CD and MD exposures (at T=0 h and T=48
440 h): as a consequence of dispersion, oil droplets have a larger surface-to-volume ratio than an
441 oil slick, and this would accelerate the solubilization of PAHs in seawater. Consideration
442 must also be given to the fact that the sum of PAHs decreased slightly in the CD and MD
443 exposure media while drastically decreased during the 48 hours of WSF exposure media. The
444 solubilization and volatilization/photolysis of PAHs are two opposing processes that
445 determine the distribution and the residence time of PAHs in seawater (Schwarzenbach et al.,
446 2003). In this case, it can be hypothesized that the dispersion of oil (CD and MD exposure)
447 triggers the solubilization of PAHs from oil droplets into the seawater, which relatively
448 compensates for the volatilization/photolysis of PAHs that occurs during the exposure.

449 Inversely the solubilization of PAHs from the oil slick to the seawater (WSF exposure) was
450 not high enough to compensate for the loss of PAHs due to volatilization/photolysis. Another
451 explanation could be that PAH loss is due to absorption by golden grey mullets as it is
452 suggested in literature for other organisms (Le Floch et al. 2003; Goanvec et al. 2008).

453 With regards to the 16 USEPA PAHs (alkylated and parent), the results show that light PAHs
454 (two to three rings) were predominant in the WSF, CD and MD exposure media at T=0 h and

455 T=48 h. This observation is consistent with the current theory that the aqueous solubility
456 increases as the molecular weight of PAHs decreases (Neff, 1979). Moreover, with the
457 exception of fluorene, the concentrations of light PAHs decreased during the experiment
458 while the concentration of heavy PAHs remained stable (cf. Indeno[1,2,3-*cd*]pyrene and
459 Dibenzo[*a,h*]anthracene in **Table 2**), a phenomenon probably attributable to the
460 volatilization/photolysis of light PAHs (Schroeder and Lane, 1988).

461

462 4.2. Fixed wavelength fluorescence analysis of biliary PAH metabolites

463

464 The fixed wavelength fluorescence of fish biliary metabolites has been used as a PAHs
465 exposure biomarker in many studies (Aas et al., 2000; Barra et al., 2001; Kopecka-Pilarczyk
466 and Correia, 2009; Insausti et al., 2009).

467 In our study pyrene-derived fluorescence was significantly higher under MD and CD
468 exposures than under control exposure (C). However, only fluorescence under CD exposure
469 was significantly higher than WSF and D exposures, which show that the exposure to pyrene
470 was higher when the oil was chemically dispersed. These results are consistent with the
471 alkylated fluoranthenes/pyrenes seawater concentration at T=48 h since this was higher under
472 CD exposure. However at T=0 h, no pyrene (alkylated or parent) was detected under CD.
473 Concerning the benzo[*a*]pyrene-type metabolites, fluorescence was higher under CD and MD
474 exposures than for the other exposure groups (WSF, D, C), indicating a higher bioavailability
475 of this PAH (and its derived type). Even though the relative fluorescence was higher under
476 WSF exposure than in other conditions (D and C), the difference was not significant. These
477 results are consistent with the benzo[*a*]pyrene concentrations measured in the seawater, since
478 the concentration of this PAH was similar for CD and MD exposures and lower for WSF
479 exposure (at T=0 h and T=48 h). Benzo[*a*]pyrene is considered carcinogenic and is a radical

480 oxygen species producer through its role as a P450 mixed-function oxidase (MFO) inducer
481 (Lemaire-Gony and Lemaire, 1993). This result is of importance because it reveals the
482 potentially high toxicity of CD and MD exposures when compared to other conditions.

483 For both metabolite types, the relative fluorescence revealed a higher exposure of fish to
484 PAHs under CD exposure (compared to WSF), probably resulting from the higher PAH
485 concentrations in the seawater. The results are consistent with the literature; indeed
486 Ramachandran et al. (2004) showed that oil dispersant increases PAHs uptake by fish exposed
487 to crude oil. Moreover Jung et al. (2009) showed that hydrocarbons metabolites in bile from
488 fish exposed to crude oil treated with dispersant were significantly higher compared with fish
489 exposed to crude oil alone.

490 To the best of our knowledge no studies have been conducted in order to allow the
491 comparison between the toxicity of an oil slick dispersed with turbulent mixing energy and
492 dispersant (CD) to an oil slick dispersed only with turbulent mixing energy (MD). Even if
493 benzo[*a*]pyrene derived metabolites levels seem to be slightly lower in MD exposure than in
494 CD exposure, no significant difference was highlighted. This is in accordance with the
495 benzo[*a*]pyrene concentrations in seawater (no difference between CD and MD exposure).
496 However, for pyrene derived metabolites, while a significant difference was observed
497 between CD and WSF, no difference was observed between MD and WSF exposure. This
498 finding is in accordance with the pyrene concentration in sea water: alkylated
499 fluoranthenes/pyrenes seawater concentration (at T=48 h) was higher under CD exposure.

500

501 4.3. EROD (7-ethoxy-resorufin-O-deethylase) activity

502

503 Since the eighties, EROD activity is commonly used to reveal PAHs biotransformation
504 (Addison and Payne, 1987) and thereby a large body of literature permits comparison of our

505 results to other studies. Furthermore, since EROD activity is involved in phase I
506 biotransformation of xenobiotics, the modulation of this biomarker in response to PAHs is
507 more precociously observed than the increase of PAHs biliary metabolites (described above).
508 By the way EROD activity measurement gives an idea of organism short term defence against
509 the xenobiotics.

510 Ramachandran et al. (2004) and Jung et al. (2009) showed an increase of EROD activity
511 following chemically dispersed oil exposure. However, our study did not show an EROD
512 activity increase while a PAHs biliary metabolites increase was observed following dispersed
513 crude oil exposure. A reason for this lack of significance could be the low sensitivity to PAHs
514 of EROD activity, when compared to biliary metabolites sensitivity (Camus et al., 1998).

515

516 4.4. Total glutathione content

517

518 The results obtained for total glutathione content in the liver of *Liza aurata* after 48 h
519 confirmed previous results concerning biliary metabolites contents since a significant
520 difference was found between dispersed oil exposure (CD and MD) and the control condition.
521 These results are consistent with the literature since Almroth et al. (2008) showed a
522 significance decrease in total glutathione in corkwing wrasse (*Symphodus melops*) exposed to
523 contaminated PAHs sites. The total glutathione content, which corresponds to reduced plus
524 oxidized glutathione (GSH+GSSG), was lower in both conditions (CD and MD), although
525 GST activity did not change. This finding shows that depletion was not due to glutathione
526 conjugation (phase II detoxification) since an increase in GST should be concomitant with
527 conjugation. Nevertheless, it is possible that the decrease in total glutathione was due to
528 inhibition of the GSH synthesis rate by contaminants, as suggested in Canesi et al. (1999), in
529 Wang et al. (2008) and in Zhang et al. (2004) on freshwater crabs, mussels and goldfish,

530 respectively. Another explanation could be that, in the process of detoxification, reduced
531 glutathione chelated the heavy metals contained in petroleum (mainly vanadium and nickel)
532 so that GS-V or GS-Ni binding complexes are formed (Sies, 1999). These complexes cannot
533 be assessed through biochemical analysis and contributed to the observed reduction in total
534 glutathione content. However, according to low heavy metals concentration in common crude
535 oil (e.g. 109.9 mg/L of Vanadium and 71.5 mg/L of Nickel, Salra Amoli et al., 2006) and the
536 short exposure period of our experiment, this explanation seems to be less accurate.

537 So, although the mechanism is not fully understood, this study shows that total glutathione is
538 depleted, suggesting, for CD and MD exposures, a reduction in the first line cellular defence,
539 since glutathione is involved in several detoxification reactions. Indeed, conjugation of
540 glutathione to contaminants can prevent them from interacting deleteriously with other
541 cellular components, enabling the organism to cope with the contaminated environment
542 (Maracine and Segner, 1998).

543 Moreover Ringwood and Connors (2000) showed that gonadal depletion of glutathione
544 induces a decrease in reproductive success in oyster. Even if this study was conducted in
545 oyster, this finding suggests that a link between the total glutathione pool and the organism
546 fitness could exist. Since our study demonstrated a depletion of the total glutathione pool in
547 the liver of juveniles golden grey mullets, it would also be interesting to assess the total
548 glutathione in the gonads of adult fish.

549

550 4.5 Antioxidant enzyme activity and lipid peroxidation (LPO).

551

552 Antioxidant enzyme activity has been shown to be modulated in response to short term (≤ 48
553 h) contaminants exposure in different targets organs of fish (Ahmad et al., 2005; Sun et al.,
554 2006; Modesto and Martinez, 2010) and especially to short term PAHs exposure in the liver

555 of golden grey mullet (Oliveira et al., 2008). However, in our study, results concerning
556 antioxidant enzyme activity showed no significant differences between exposure conditions,
557 suggesting that oxidative stress was absent.

558 LPO was measured via the malondialdehyde content in the liver and revealed the targeting of
559 cell membranes by radical oxygen species (ROS), thus altering membrane fluidity,
560 compromising membrane integrity, inactivating membrane-bound enzymes and disrupting
561 surface receptor molecules. In Ahmad et al. (2005) and in Oliveira et al (2008), a LPO
562 increase was observed in fish gills after 48 h of contamination and in fish livers after 16 h of
563 contamination, respectively. In our study the high intragroup variability, when compared to
564 other studies (Oliveira et al., 2008; Kopecka-Pilarczyk and Correia, 2009), induced a lack of
565 significance, confirming the notion that oxidative stress was absent. However it should be
566 stated that, for exposure conditions containing petroleum (CD, MD, WSF) a high intragroup
567 variability was observed whereas a lower variability was observed following Control (C) and
568 single dispersant (D) exposure. This observation suggests a difference of oxidative stress
569 between the individuals exposed to conditions containing petroleum.

570 Oliveira et al. (2008) evaluated oxidative stress using, as in our study, LPO and antioxidant
571 enzyme activity in the liver of *Liza aurata* exposed to a PAH (phenanthrene). They found a
572 significant difference in these biomarkers, but the concentrations of phenanthrene were 50 to
573 1300 times higher than in our study.

574

575 4.6 Haemolytic activity of alternative complement pathway (ACH 50)

576

577 The innate immune function has also been used as a biomarker of PAH toxicity (Seeley and
578 Weeks-Perkins, 1997; Carlson et al., 2004). The complement system of teleost fish is a
579 powerful defence system since it is involved in important immune functions that are pivotal to

580 the recognition and clearance of microbes (Boshra et al., 2006). Moreover, the haemolytic
581 activity of the alternative complement pathway has been shown to be a suitable biomarker of
582 PAHs contamination in teleost fish (Bado-Nilles et al., 2009). On this basis, the alternative
583 complement pathway was chosen since its functional degradation by exposure to petroleum
584 compounds could reveal an alteration in fish health.

585 In our study no significant difference was found between the control condition and
586 contaminant exposures, even though the haemolytic activity seemed to be lower following
587 CD exposure. Bado-Nilles et al. (2009) found significant differences between contaminated
588 and control fish, for a sum of PAH concentrations that was lower than in our study, but for
589 longer exposure times, suggesting that alteration of haemolytic activity could have been
590 observed after more than 48 h of exposure.

591

592 **5. Conclusion**

593

594 Based on fixed wavelength fluorescence analysis of biliary PAH metabolites, the results from
595 this study show higher exposure for dispersed crude oil (CD and MD) than for other types of
596 contaminant exposure. Also, the total glutathione content, described as a first line cellular
597 defence against contaminants, was significantly reduced under dispersed oil exposures.
598 Antioxidant enzymes did not show any responses to the contamination. EROD activity, lipid
599 peroxidation and the haemolytic activity of the complement system also did not respond when
600 fish were exposed to contaminants.

601 These results demonstrate a significant response of biomarkers to chemically dispersed oil,
602 when compared to a non-dispersed oil slick (water-soluble fraction of oil), suggesting that oil
603 slicks must not be dispersed when containment and recovery can be conducted at the oil spill
604 site (low mixing energy of seawater). This finding is in accordance with an important body of

605 literature: many studies show an increase of PAHs toxicity to fish following dispersant
606 application (Perkins et al., 1973; Cohen and Nugegoda, 2000; Ramachandran et al., 2004; Lin
607 et al., 2009). On the other hand, no significant difference in the response of biomarkers was
608 observed between chemically and mechanically dispersed oil. This finding suggests that when
609 containment and recovery cannot be conducted (high mixing energy of seawater) the
610 application of dispersant in nearshore areas is no more toxic than the natural dispersion
611 (wave, current, swell).

612 To conclude, the results of this study are of interest with regards establishing a framework for
613 dispersant use and policies in nearshore areas since they are part of a current project:
614 DISCOBIOL project (DISpersant and response techniques for COastal areas: BIOLogical
615 assesment and contributions to the regulation). Initially, this project intends to assess the
616 toxicity of chemically dispersed oil to several species living in nearshore areas (*Crassostera*
617 *gigas*, *Mytilus edulis*, *Scophthalmus maximus*, *Dicentrarchus labrax* and *Liza aurata*). For this
618 reason, organisms were exposed to oil in the water column. However, since dispersed crude
619 oil can interact with other components of nearshore area habitats, such as mudflats, further
620 studies must be conducted in order to better evaluate the net environmental benefits of
621 dispersant application in nearshore areas.

622

623

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625

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632

633

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885 **Table 1:** Dispersant nominal concentration, TPHs and sum of 16 parents and alkylated US-
 886 EPA PAHs (Σ PAHs) concentration in the five exposure media at the beginning (T=0 h) and at
 887 the end of the exposure (T=48 h) to C (Control), CD (Chemically Dispersed oil), MD
 888 (Mechanically Dispersed oil), WSF (Water Soluble Fraction of oil) and D (Dispersant).
 889 Values are expressed as mean \pm standard error mean of both experimental replicates. n.d. =
 890 not detected. n.a. = not assessed.

891

	[TPHs] _{T=0h} (mg/L)	[TPHs] _{T=48h} (mg/L)	[Σ PAHs] _{T=0h} (μ g/L)	[Σ PAHs] _{T=48h} (μ g/L)	[Dispersant] _{nom.} (mg/L)
C	n.d.	n.d.	n.d.	n.d.	n.a.
CD	39.1 \pm 4.1	25.1 \pm 3.1	43.98 \pm 5.5	26.34 \pm 2.7	3.33
MD	13.15 \pm 2.6	9.30 \pm 0.2	39.09 \pm 0.6	20.63 \pm 0.1	n.a.
WSF	n.d.	n.d.	5.16 \pm 0.6	0.47 \pm 0.07	n.a.
D	n.d.	n.d.	n.d.	n.d.	3.33

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894 **Table 2:** Concentration of 16 US-EPA PAHs (alkylated and parents) in sea water during CD
895 (Chemically Dispersed oil), MD (Mechanically Dispersed oil) and WSF (Water Soluble
896 Fraction of oil) exposures. Values are expressed as mean \pm standard error mean of both
897 experimental replicates. n.d. = not detected.
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16 US-EPA PAHs (parents and alkylated)	Molecular weight (g/mol)	Concentration (ng/L) at T=0h and T=48h					
		T= 0 h			T=48 h		
		CD	MD	WSF	CD	MD	WSF
Naphtalene	128.2	2287 \pm 78	1842 \pm 101	311 \pm 10	335 \pm 14	262 \pm 6	32 \pm 5
C1-Naphtalene	143.2	6936 \pm 1699	7569 \pm 49	987 \pm 22	3244 \pm 61	2658 \pm 53	78 \pm 22
C2-Naphtalene	158.2	15579 \pm 199	12766 \pm 223	1668 \pm 172	7937 \pm 393	6396 \pm 46	95 \pm 9
C3-Naphtalene	173.2	11496 \pm 385	9957 \pm 59	1298 \pm 183	7677 \pm 491	6506 \pm 47	59 \pm 6
C4-Naphtalene	188.2	4488 \pm 129	4081 \pm 21	450 \pm 57	3696 \pm 106	3094 \pm 226	64 \pm 12
Acenaphtylene	152.2	27 \pm 3	16 \pm 3	n.d.	n.d.	n.d.	n.d.
Acenaphtene	154.2	n.d.	n.d.	n.d.	n.d.	n.d.	1 \pm 0
Fluorene	166.2	241 \pm 3	196 \pm 8	53 \pm 9	400 \pm 298	89 \pm 2	1 \pm 0
C1-Fluorene	181.2	336 \pm 5	291 \pm 4	70 \pm 11	663 \pm 480	158 \pm 2	4 \pm 1
C2-Fluorene	196.2	316 \pm 3	284 \pm 9	44 \pm 7	734 \pm 523	187 \pm 3	3 \pm 0
C3-Fluorene	211.2	169 \pm 2	130 \pm 17	16 \pm 1	329 \pm 231	57 \pm 31	3 \pm 0
Phenanthrene	178.2	316 \pm 300	522 \pm 16	79 \pm 4	160 \pm 151	241 \pm 1	5 \pm 0

Anthracene	178.2	3±0	8±8	2±1	n.d.	6±6	n.d.
C1-Phenanthrenes/Anthracene	193.2	959±32	818±17	66±23	569±18	467±3	5±0
C2-Phenanthrenes/Anthracene	208.2	489±4	390±25	34±4	326±2	295±9	n.d.
C3-Phenanthrenes/Anthracene	223.2	136±1	89±0	8±1	75±0	68±2	n.d.
C4-Phenanthrenes/Anthracene	238.2	36±5	29±4	n.d.	23±1	17±3	n.d.
Fluoranthene	202.3	n.d.	2±2	1±0	2±0	1±1	n.d.
Pyrene	202.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C1-Fluoranthenes/Pyrenes	217.3	n.d.	n.d.	n.d.	n.d.	3±3	n.d.
C2-Fluoranthenes/Pyrenes	232.3	n.d.	7±7	n.d.	9±1	5±5	n.d.
C3-Fluoranthenes/Pyrenes	247.3	n.d.	4±4	n.d.	3±3	3±3	n.d.
Benzo[<i>a</i>]anthracene	228.3	n.d.	n.d.	n.d.	1±0	n.d.	n.d.
Chrysene	228.3	8±8	9±9	6±3	27±8	19±5	14±2
Benzo[<i>b+k</i>]fluoranthene	252.3	3±1	6±0	5±0	10±6	8±3	9±1
Benzo[<i>a</i>]pyrene	252.3	3±0	3±0	2±0	5±1	5±0	4±1
Benzo[<i>g,h,i</i>]perylene	276.3	34±5	3±3	32±3	4±1	3±1	4±4
Indeno[<i>1,2,3-cd</i>]pyrene	276.3	51±31	31±0	3±0	49±0	37±0	40±0
Dibenzo[<i>a,h</i>]anthracene	278.4	63±3	39±3	25±1	57±4	45±2	48±4

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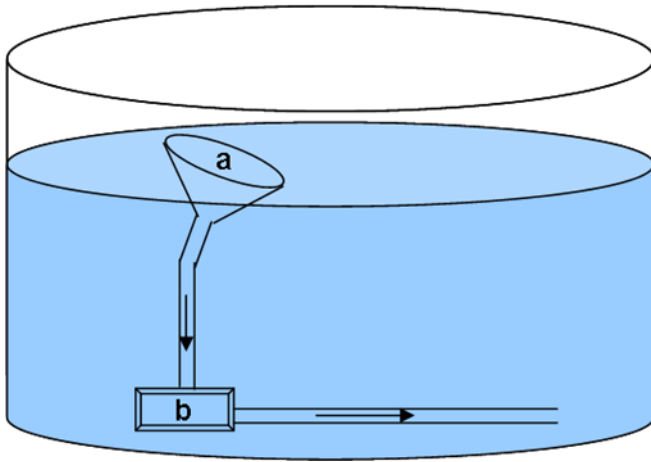
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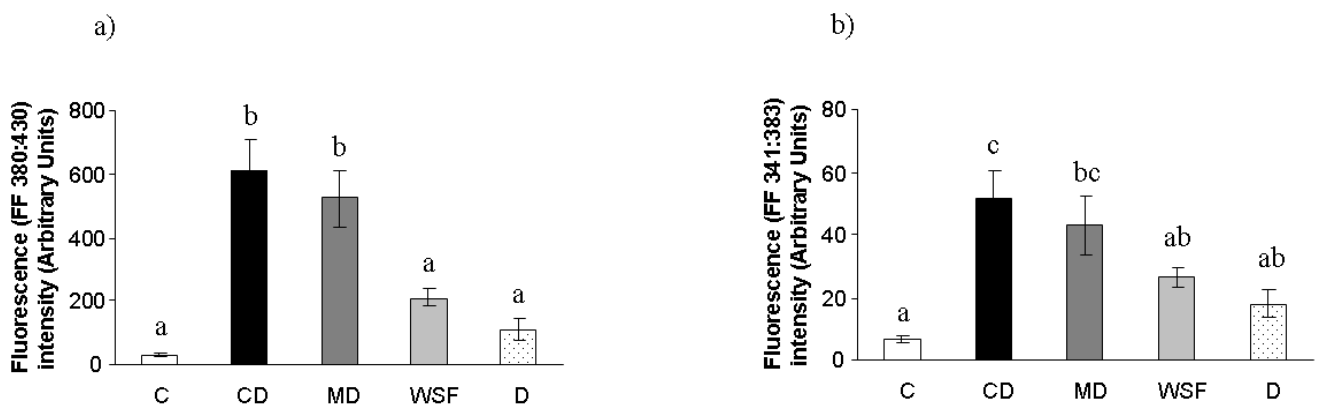
908 **Figure 1:** The experimental system constituted of a funnel (a) linked to a water pump (b) in a

909 300-l sea tank. (→) indicates the direction of seawater and/or contaminants through the

910 experimental system

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914 **Figure 2:** Fixed wavelength fluorescence (FF) of bile reflecting biliary PAHs metabolites

915 levels after 48 h exposure to Control solution (C), Chemically Dispersed oil solution (CD),

916 Mechanically Dispersed oil solution (MD), Water Soluble Fraction solution (WSF) and

917 Dispersant solution (D): (a) FF 380:430 (benzo[a]pyrene type of metabolites); (b) FF 341:383

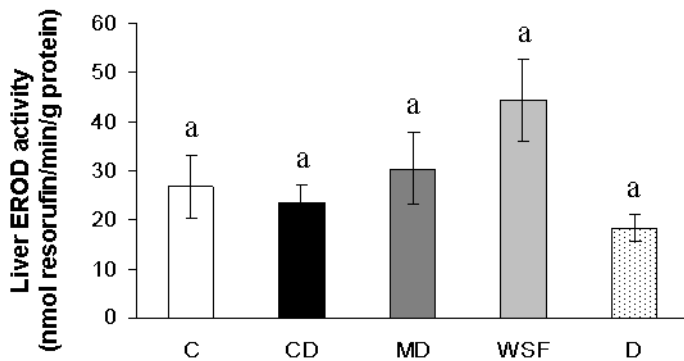
918 (pyrene derived type of metabolites). Levels are expressed as fluorescence intensity. Values

919 represent mean \pm standard error (n=10 per treatment). Different letters above bars indicate a

920 significant difference, where $P < 0.05$.

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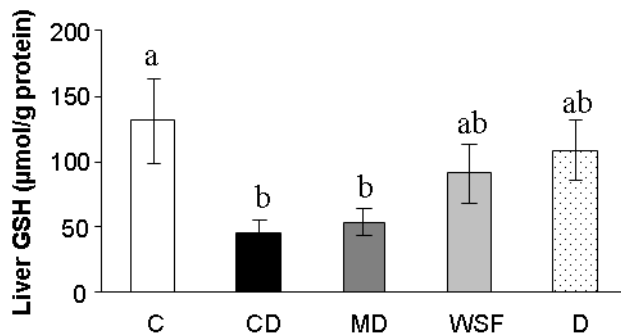
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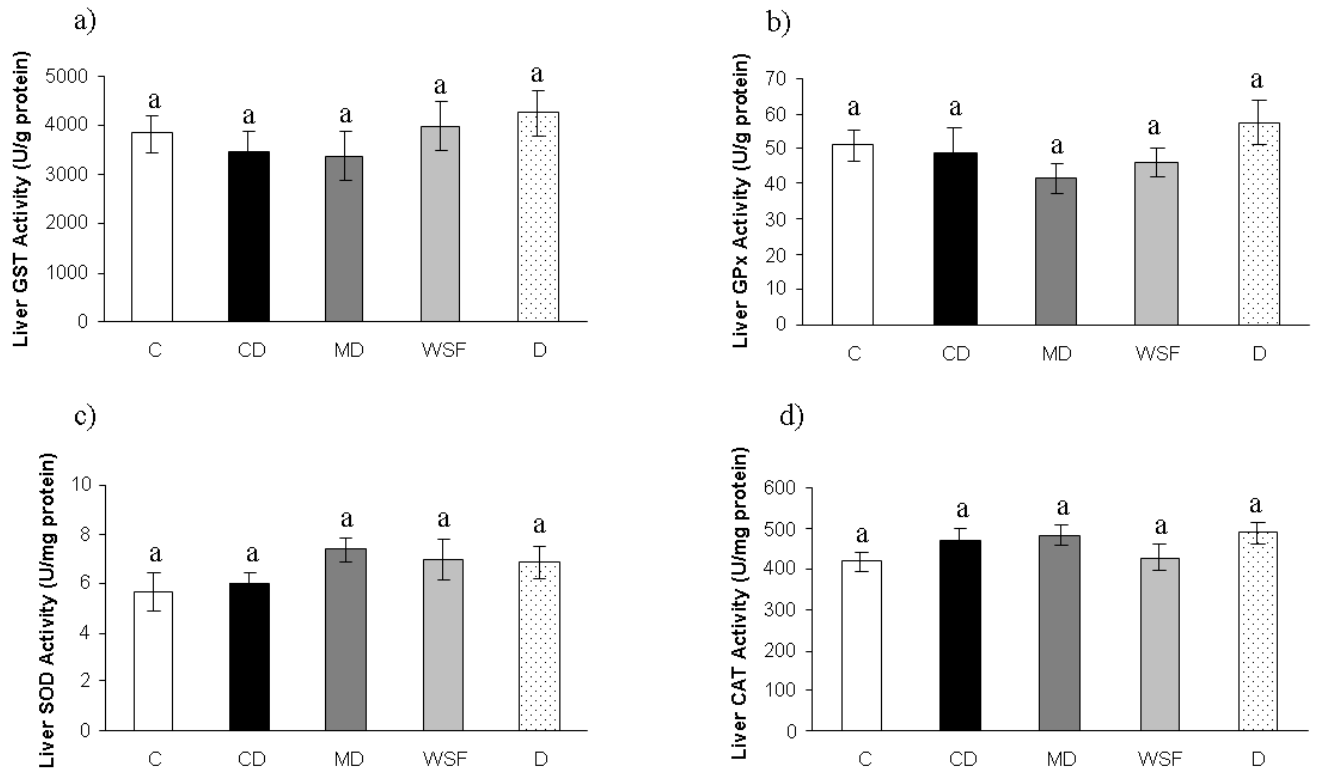
924 **Figure 3:** EROD (7-ethoxy-resorufin-O-deethylase) activity in *Liza aurata* after 48 h
925 exposure to Control solution (C), Chemically Dispersed oil solution (CD), Mechanically
926 Dispersed oil solution (MD), Water Soluble Fraction (WSF) solution and Dispersant solution
927 (D). Values represent mean \pm standard error (n=10 per treatment). Different letters above bars
928 indicate a significant difference, where $P < 0.05$.

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930

931 **Figure 4:** Total glutathione (GSH) content in liver of *Liza aurata* after 48 h exposure to
932 Control solution (C), Chemically Dispersed oil solution (CD), Mechanically Dispersed oil
933 solution (MD), Water Soluble Fraction (WSF) solution and Dispersant solution (D). Values
934 represent mean \pm standard error (n=10 per treatment). Different letters above bars indicate a
935 significant difference, where $P < 0.05$.

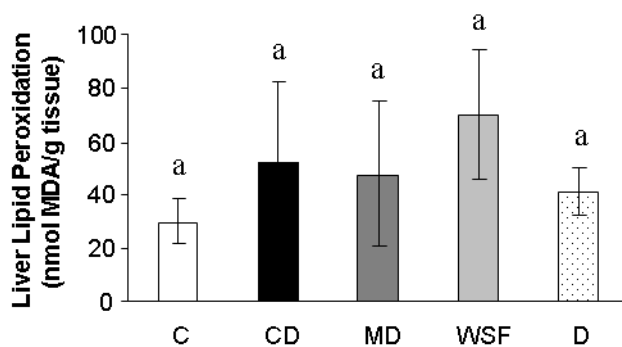


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937 **Figure 5:** a) Glutathione S-Transferase (GST) activity, b) Glutathione Peroxidase (GPx)
 938 activity, c) Superoxide Dismutase (SOD) activity and d) Catalase (CAT) activity in liver of
 939 *Liza aurata* after 48 h exposure to Control solution (C), Chemically Dispersed oil solution
 940 (CD), Mechanically Dispersed oil solution (MD), Water Soluble Fraction (WSF) solution and
 941 Dispersant solution (D). Values represent mean \pm standard error (n=10 per treatment).

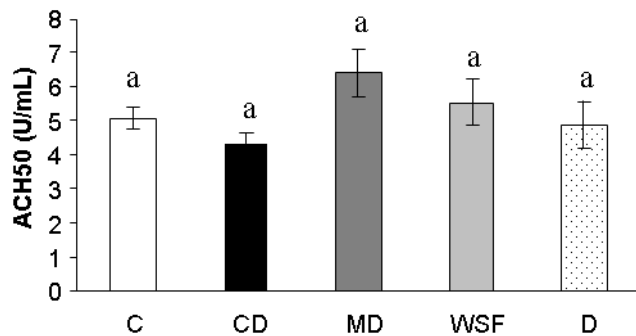
942 Different letters above bars indicate a significant difference, where $P < 0.05$.

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945 **Figure 6:** Lipid peroxidation in liver of *Liza aurata* after 48 h exposure to Control solution
946 (C), Chemically Dispersed oil solution (CD), Mechanically Dispersed oil solution (MD),
947 Water Soluble Fraction (WSF) solution and Dispersant solution (D). Values represent mean \pm
948 standard error (n=10 per treatment). Different letters above bars indicate a significant
949 difference, where $P < 0.05$.
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951
952 **Figure 7:** Haemolytic activity of alternative complement pathway (ACH 50) in plasma of
953 *Liza aurata* after 48 h exposure to Control solution (C), Chemically Dispersed oil solution
954 (CD), Mechanically Dispersed oil solution (MD), Water Soluble Fraction (WSF) solution and
955 Dispersant solution (D). Values represent mean \pm standard error (n=10 per treatment).
956 Different letters above bars indicate a significant difference, where $P < 0.05$.
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958