Toxicity of dispersant application: biomarkers responses in gills of juvenile golden grey mullet (Liza aurata)

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27   **Abstract**

28

29   Dispersant use in nearshore areas is likely to increase the exposure of aquatic organisms to

30   petroleum. To measure the toxicity of this controversial response technique, golden grey

31   mullets (*Liza aurata*) were exposed to mechanically dispersed oil, chemically dispersed oil,

32   dispersant alone in seawater, water-soluble fraction of oil and to seawater as a control

33   treatment. Several biomarkers were assessed in the gills (enzymatic antioxidant activities,

34   glutathione content, lipid peroxidation) and in the gallbladder (polycylic aromatic

35   hydrocarbons metabolites). The significant differences between chemically dispersed oil and

36   water soluble fraction of oil highlight the environmental risk to disperse an oil slick when

37   containment and recovery can be conducted. The lack of significance between chemically and

38   mechanically dispersed oil suggests that dispersant application is no more toxic than the

39   natural dispersion of the oil slick. The results of this study are of interest in order to establish

40   dispersant use policies in nearshore areas.

41

42   *Capsule*:

43   When the meteorological conditions induce the dispersion of the oil slick (e.g. wave), the

44   application of dispersant does not increase the toxicity of petroleum.

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1. Introduction

Dispersant application is an oil spill response technique that permits the transfer of the oil slick from the surface to the water column. When applied on an oil slick, the chemical formulation of the dispersant (surface active agent) induces the formation of oil–surfactant micelles. In offshore areas, dispersion shows many advantages since it accelerates the bacterial degradation of petroleum (Tiehm, 1994; Churchill et al., 1995), reduces the chance of drifting of the oil slick to the shoreline and also limits the risk of contamination to the surface occupying organisms (e.g. seabirds, marine mammals). However, when the oil spill site is in a nearshore area or if an oil slick reaches the coast (as observed recently in the Deep Water Horizon oil spill), slick dispersion is prohibited (Chapman et al., 2007). This environmental precautionary principle is based (i) on the low dilution potential of the oil slick in the shallow waters of nearshore areas and (ii) on the ecological sensitivity of nearshore areas since they are nurseries for many fish species (Martinho et al., 2007). On the other hand, a field study conducted by Baca et al. (2006), but only applicable to nearshore mangroves, seagrass and coral ecosystems, revealed a positive net environmental benefit of dispersant application in nearshore areas. Thus, with regards to the precautionary principle and the recent results of field studies, dispersant application in nearshore areas seems to be a controversial response technique. In this context, in order to contribute to dispersant use policies in nearshore areas, an on-going project is being conducted: the DISCOBIOL project (DISpersant and response techniques for COastal areas; BIOLogical assessment and contributions to the regulation). This study is part of this project and intended to assess the toxicity of a chemically dispersed oil. For this purpose, most studies have evaluated the toxicity of dispersant alone in seawater (Adams et al., 1999; George-Ares and Clark, 2000) or the dispersed oil water-accommodated fraction (Cohen and Nugegoda, 2000; Mitchell and

77 Holdway, 2000; Ramachandran et al., 2004; Perkins et al., 2005; Jung et al., 2009), not taking

78 into account the presence of oil droplets in the water column. However, many field

79 observations have shown the presence of oil droplets in the water column. Their formation

80 can be induced within 2 hours (Cormack, 1977) or during a period of more than 1 week, as

81 observed during the Braer oil spill (Lunel, 1995). In this context, our experimental approach

82 was conducted in order to evaluate the actual toxicity of a chemically dispersed oil treatment

83 containing oil droplets. Toxicity was measured through the assessment of biomarkers in a

84 target organ of a pelagic fish species.

85 Oxidative stress and antioxidant defences were considered as suitable biomarkers since they

86 have been shown in many studies to respond to petroleum contamination and especially to the

87 PAH (polycyclic aromatic hydrocarbons) contained in petroleum (Avci et al., 2005 ; Almroth

88 et al., 2008; Oliveira et al., 2008; Jung et al., 2009; Kopecka Pylarczyk and Correia, 2009;

89 Narghang et al., 2009). Moreover, both, oxidative stress and antioxidant defences could give

90 information on the health of the contaminated organisms: (i) oxidative stress, since it is

91 considered as a cause of tissue injury (Halliwell, 1999) and (ii) antioxidant defences, since

92 authors linked modulation of this biological parameter to fish health indicators such as

93 progression of diseases and/or cellular mortality (Allen and Moore, 2004).

94 In our study, oxidative stress and antioxidant defences were assessed by evaluating lipid

95 peroxidation (a marker of lipid degradation due to oxidative stress) and evaluating the

96 response of antioxidant enzymatic activities (catalase, superoxide dismutase and glutathione

97 peroxidase), respectively.

98 Additionally, total glutathione was measured taking into account the importance of the

99 cellular status of this molecule for the defence of the organism against xenobiotics (Maracine

100 and Segner, 1998). Indeed, glutathione is implied in many cellular defence mechanisms such

101 as (i) antioxidant defences, by its conjugation to reactive oxygen species (Amiard-Triquet and

4

102 Amiard, 2008); (ii) heavy metals (such as Vanadium and Nickel present in petroleum, Salar

103 Amoli et al., 2006) chelation, as described in Sies, 1999; and (iii) detoxification processes, by

104 its conjugation to xenobiotics such as PAH (van der Oost et al., 2003). In our study, we

105 evaluated the glutathione status through the measurement of the total glutathione content

106 which is the sum of the oxidized and the reduced form of this molecule.

107 These biomarkers were assessed in fish gills, taking into account their target organ status:

108 several studies have shown an effect of petroleum compounds on gills (McKeown, 1981;

109 Oliveira et al., 2008; Mendonça Duarte et al., 2010). In parallel, PAH biliary metabolites were

110 measured in order to evaluate the level of exposure to PAH following dispersant application.

111 The choice of the golden grey mullet (*Liza aurata*) as a biological model was based on the

112 fact that (i) it represents a relevant biomass in nearshore ecosystems; (ii) it is a commercially

113 important species especially in Europe and Egypt (Gautier and Hussenot, 2005) and (iii) this

114 species is present in nearshore areas during its early life stages (Gautier and Hussenot, 2005)

115 being consequently a target organism for anthropogenic pollutants (Bruslé, 1981).

116

117 **2. Materials and methods**

118

119 2.1. Experimental design

120

121 2.1.1. Experimental animals

122

123 Fifty juvenile golden grey mullets (*Liza aurata*), fished in Venice (Italy) lagoons and

124 provided by Commercio Pesca Novellame Srl (Chioggia, Italy), were used to conduct this

125 study.

126 For 4 weeks, fish were acclimatized in 300-L flow-through tanks prior to the exposure studies

127 (dissolved oxygen: 94 ± 2%; salinity: 35 ± 0%; temperature: 14.9 ± 0.5 °C, with a 12 h

128 light:12 h dark photoperiod in seawater free of nitrate and nitrite). During acclimation, they

129 were fed daily with fish food (Neosupra AL3, from Le Gouessant aquaculture) which does

130 not contain additives (also called synthetics) antioxidants authorised by the European Union

131 (butyl-hydroxy-anisol, butyl-hydroxy-toluene, ethoxyquin, propyl gallate and octyl gallate).

132 Fish were starved for 48 h prior to bioassays and throughout the exposure period, in order to

133 avoid bile evacuation from the gallbladder. Prior to bioassay, their average length was 136.6 ±

134 0.1 mm (mean ± standard error of the mean) and their average weight was 32.33 ± 0.87 g.

135

136 2.1.2 Chemicals

137

138 A dispersant formulation (Total Fluides) was selected based on its efficiency. The efficiency

139 was preliminary determined in the CEDRE (CEntre de Documentation de Recherche et

140 d'Expérimentations sur les pollutions accidentelles des eaux, France) using the method

141 NF.T.90-345. The dispersant was non-toxic at the concentration recommended by the

142 manufacturer (preliminary determined using standard toxicity test: method NF.T.90-349) and

143 biodegradable.

144 A Brut Arabian Light (BAL) crude oil was selected for this study. The oil is composed of

145 54% saturated hydrocarbons, 10% polar compounds and 36% aromatic hydrocarbons.

146 To simulate the natural behaviour of the oil after it is released at sea (evaporation of light

147 compounds and natural photodegradation, respectively) the oil was evaporated under

148 atmospheric conditions and natural UV-sunlight, prior to fish exposure. The resulting

149 chemical composition of the oil was 54% saturated hydrocarbons, 12% polar compounds and

150 34% aromatic hydrocarbons. Among aromatic hydrocarbons, concentration of 21 PAH was

151   measured (the 16 PAH listed by the USEPA as priority pollutants and five supplementary

152   PAH: benzo[*b*]thiophene, biphenyl, dibenzothiophene, benzo[*e*]pyrene, perylene). The sum of

153   the 21 PAH represents 16.4 mg/g of petroleum (1.64 % of the petroleum). More information

154   concerning the composition of the petroleum used in this study is available in (Milinkovitch et

155   al., accepted for publication).

156

157   2.1.3. Experimental system (**Figure 1**)

158

159   The experimental system (also described in Milinkovitch et al., 2011) comprised five 300-L

160   seawater tanks. Each one contains a funnel (**a,** at the surface) linked to a Johnson L450 water

161   pump (**b,** at the bottom of the tank). After 24 h homogenization, this system was set up to

162   maintain a mixture of oil and dispersant as a homogenous solution despite the hydrophobic

163   character of the oil (preliminary tests not shown). The temperature in this static water system

164   was controlled using two heaters (RENA CAL 300) so that the exposure temperature was

165   15.3 ± 0.3 °C (mean ± standard error mean). Other physico-chemical parameters were also

166   measured: seawater was free of nitrate and nitrite, pH (7.99 ± 0.03) and dissolved oxygen (98

167   ± 5%) remained constant throughout the study.

168

169   2.1.4. Experimental treatments

170

171   Each experimental tank contained 300 L seawater provided by Oceanopolis (France). The

172   control treatment (C) was made up using 300 L seawater. The chemically dispersed (CD) oil

173   treatment was made by pouring 20 g of petroleum and 1 g of dispersant into the funnel of the

174   experimental system. The mechanically dispersed (MD) oil treatment was made by pouring

175   20 g of petroleum into this funnel. The dispersant alone (D) treatment, as an internal control

176 of CD, was made by pouring 1 g of dispersant into the funnel. For the water-soluble fraction

177 of oil (WSF), a 20 g oil slick was contained using a plastic cylinder (21 cm diameter) placed

178 on the surface of the seawater (in addition to the funnel and the pump, which were kept to

179 maintain the same level of agitation of the seawater as for other treatments). Readers must

180 take into account that the spreading of the oil slick was not prevented by the plastic cylinder

181 since the oil slick was smaller than the diameter of the plastic cylinder. Thereby the

182 experimental approach simulates the actual spreading behaviour of oil at sea. During the

183 entire exposure period, the oil slick remained at the surface without mixing. No droplet was

184 observed in the water column (visual observations) suggesting that the fish were only exposed

185 to the soluble fraction of the oil.

186 While the solutions remained homogenous (less than 5 % difference between three TPH

187 concentration measurements sampled at three differents depths in the experimental tanks), no

188 fish were exposed for 24 hours after making up the solutions. Then, groups of 10 fish were

189 randomly distributed in the five experimental tanks, each tank containing an exposure media

190 (described above). The fish were exposed for 48 h (from T=0 h to T=48 h).

191 At the end of the exposure period, the fish in each tank (each treatment) were euthanized

192 using eugenol (99 %, Sigma Aldricht chemicals, France). The gallbladder was removed from

193 each fish and stored at -80 °C prior to analysis. Gills were rinsed off by dipping them in PBS

194 (Phosphate Buffered Sodium 0.01 M, pH=7.4, Sigma) in order to remove blood. Then, the

195 gills were homogenized in another PBS solution. The homogenates were centrifuged at

196 10,000 g, 4 °C, for 15 min to obtain the post-mitochondrial supernatant. Total protein

197 concentrations in supernatants were determined using the method of Bradford (1976) with

198 bovine serum albumin (Sigma-Aldrich Chemicals, France). Then, supernatants were stored at

199 -80 °C prior to biochemical analysis.

200

201

202   2.2. Total petroleum hydrocarbon (TPH) seawater concentrations

203

204   The TPH concentration, which is the sum of the dissolved hydrocarbon concentrations plus

205   the amount of oil droplets, was measured for all treatments at the beginning (T=0 h) and at the

206   end of fish exposure (T=48 h), using the mean of three replicated measurements for each time

207   point. The samples were extracted with 10 mL of dichloromethane (Carlo Erba Reactifs,

208   SDS). After separation of the organic and aqueous phases, water was extracted two additional

209   times with the same volume of dichloromethane (2 x 10 mL). The combined extracts were

210   dried on anhydrous sulphate and then analysed using a UV spectrophotometer (UV-Vis

211   spectrophometer, Unicam, France) at 390 nm, as described by Fusey and Oudot (1976).

212   Assays were conducted in collaboration with Cedre (Centre de Documentation de Recherche

213   et d'Expérimentations sur les Pollutions Accidentelles des Eaux), a laboratory with agreement

214   ISO 9001 and ISO 14001. In accordance with Cedre, results are not reliable under 1mg/L.

215

216   2.3. Biochemical analysis

217

218   2.3.1. Fixed wavelength fluorescence analysis of bile

219

220   Bile samples were diluted (1:1000) in absolute ethanol (VWR International, France) and

221   assessments were conducted for three fixed wavelength fluorescence (FF). FF 290:335 mainly

222   detects naphthalene-derived metabolites, FF 341:383 mainly detects pyrene-derived

223   metabolites and FF 380:430 mainly detects benzo[*a*]pyrene-derived metabolites (Aas et al.,

224   2000). Measurements were performed in quartz cuvettes (Sigma Aldricht, USA) on a

225   spectrofluorimeter (SAFAS Flx-Xenius, Monaco). The FF values were expressed as arbitrary

226   units of fluorescence and the signal level of pure ethanol was subtracted. Depending on the

227   spectrofluorimeter, results are not reliable under 0.1 arbitrary units.

228

229   2.3.2. Total glutathione (GSH)

230

231   Total (reduced plus oxidized) glutathione was determined spectrophotometrically in gills,

232   according to the procedure of Akerboom and Sies (1981) and using a glutathione assay kit

233   (SIGMA CS0260, Sigma Aldricht, USA). The samples were first deproteinized with 5% 5-

234   sulfosalicylic acid solution. The glutathione content of the sample was then assayed using a

235   kinetic assay in which amounts of glutathione cause a continuous reduction of 5,5′-dithiobis-

236   (2-nitrobenzoic) acid (DTNB) to TNB. The oxidized glutathione formed was recycled by

237   glutathione reductase and NADPH. The product, TNB, was assayed colorimetrically at 412

238   nm in UV microplates (Greiner Bio One), using a spectrophotometer (SAFAS Flx-Xenius,

239   Monaco). The results are presented in µmol of GSH/g of protein.

240

241   2.3.3. Antioxidant enzymes and lipid peroxidation

242

243   Glutathione peroxidase (GPx) activity was determined according to the method of Paglia and

244   Valentine (1967), using a glutathione peroxidase assay kit (RS504/RS 505, RANDOX,

245   France). GPx catalyses the oxidation of reduced glutathione by cumene hydroperoxide. In the

246   presence of glutathione reductase and NADPH the oxidized glutathione (GSSG) is

247   immediately converted to the reduced form with concomitant oxidation of NADPH to

248    $NADP^+$. The decrease in absorbance was measured at 340 nm.

249   Superoxide dismutase (SOD) activity was determined according to the method of Wooliams

250   et al. (1983) and using a superoxide dismutase assay kit (SD125, RANDOX, France). This

251 method employs xanthine and xanthine oxidase to generate superoxide radicals which react

252 with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red

253 formazan dye, assessed at 505 nm. The superoxide dismutase activity was then measured by

254 the degree of inhibition of this reaction. One unit of SOD was that which causes a 50%

255 inhibition of the rate of reduction of INT. The results are presented in units of SOD/mg of

256 protein.

257 Catalase (CAT) activity was determined according to the method of Deisseroth and Dounce

258 (1970) and using a catalase assay kit (CAT 100, Sigma Aldricht, USA). Samples were mixed

259 (v:v) with hydrogen peroxide. The kinetics of hydrogen peroxide degradation were assessed

260 at 280 nm. The results are expressed in units of CAT/mg of protein.

261 Lipid peroxidation levels were assessed via malondialdehyde (MDA) contents determined

262 using a commercially available MDA assay kit (MDA assay kit, Oxis International, USA).

263 The method was based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole,

264 with MDA. The blue product was quantified by measuring absorbance at 586 nm (Gérard-

265 Monnier et al., 1998). The results are presented in nmol of MDA/g of tissue.

266 Materials used to measure spectrophotometrically antioxidant enzymes activity and lipid

267 peroxidation were UV microplates (Greiner Bio One, Germany) and a spectrophotometer

268 (SAFAS Flx-Xenius, Monaco).

269

270 2.4. Statistical analysis

271

272 The statistical analysis was carried out using XLstat 2007 software. The assumptions of

273 normality and homoscedasticity were verified using the Kolmogorov-Smirnov and Cochran

274 tests, respectively. When homoscedasticity and normality were not respected, a Kruskal

275 Wallis test was conducted to highlight significant differences between treatments. When

276 homoscedasticity and normality were respected, a factorial analysis of variance (one-way

277 ANOVA) was performed in order to assess the effects of the different treatments. This

278 statistical analysis was followed by the Tukey post-hoc test to detect significant differences

279 between groups. Correlations between fixed wavelength fluorescence intensity and other

280 variables (GSH, GPx, SOD, CAT and LPO) were conducted using the Spearman test. The

281 significance of the results was ascertained at $\alpha=0.05$. The results are expressed as means $\pm$

282 s.e.m. (standard error of the mean) corresponding to groups of 10 fish (n=10).

283

284

285 **3. Results**

286

287 No fish died during the acclimation and exposure period. TPH were not detected in the

288 Control (C) and Dispersant (D) treatments. Moreover no oil slick was observed in the

289 Chemically Dispersed oil (CD) and the Mechanically Dispersed oil (MD) treatments. In the

290 Water Soluble Fraction of oil (WSF) treatment, the oil slick remained at the surface

291 throughout the exposure period and no droplets were observed (visual observations) in the

292 water column.

293

294 3.1. Total petroleum hydrocarbons (TPH)

295

296 The concentration of TPH (**Table 1**) was slightly higher in the CD than in the MD treatment

297 at T=0 h and at T=48 h. A 68% decrease was observed in the CD treatment (from 46.4 to 14.9

298 mg/L) and a 73% decrease was observed in the MD treatment (from 39.4 to 10.7 mg/L)

299 during the 48 h exposure period. No TPH were detected in the WSF treatment, probably due

300 to the detection limit of the method.

301

302 3.2. Fixed wavelength fluorescence analysis of bile

303

304 Whatever the fixed wavelength employed (**Figure 2**), no significant difference was found

305 between the fluorescence intensity of the WSF, D and C treatments.

306 Whatever the fixed wavelength employed, the fluorescence intensity was significantly higher

307 in the CD treatment than in the C, D and WSF treatments.

308 At FF 380:430 and FF 343:383, the fluorescence intensity was higher in the MD treatment

309 than in the C, D and WSF treatments whereas no significant difference was found at FF

310 290:335.

311 At FF 290:335 and FF 343:383, the fluorescence intensity was lower in the MD treatment

312 than in the CD treatment whereas no significant difference was observed at FF 380:430.

313

314 3.3. Total glutathione (GSH)

315

316 Gill GSH content (**Figure 3**) was significantly lower in the CD than in the C, D and WSF

317 treatments, whereas no significant difference was observed between the CD and MD

318 treatments. No significant difference was observed between MD and the other treatments (C,

319 D and WSF).

320 Significant correlations were found between the fluorescence intensities FF 343:383 and FF

321 380:430 with GSH content (P= 0.001 and P=0.002 respectively) whereas there was no

322 correlation between the fluorescence intensity at 290:335 with GSH content (P>0.05).

323

324 3.4. Antioxidant enzymatic activity and lipid peroxidation (LPO)

325

326 No significant difference was found between the five treatments (P>0.05), in terms of

327 antioxidant enzymatic (SOD, CAT, GPx) activities (**Figure 4**). With regards to SOD activity,

328 the lack of significance could be due to the high intragroup variability. With regards to GPx,

329 the enzymatic activity seemed to be higher in the CD treatment than in the other treatments.

330 No correlation was found between the enzymatic activities and fixed wavelength fluorescence

331 intensity (P>0.05). There was no significant difference in LPO (**Figure 5**) between the five

332 treatments (P>0.05) and no correlation was found between LPO and the fixed wavelength

333 fluorescence intensities (P>0.05).

334

335 **4. Discussion**

336

337 The aim of this study was to evaluate the toxicity of dispersant application. Through an

338 experimental approach, several scenarios occurring during an oil spill were considered and

339 their toxicity was evaluated. Five exposure treatments were conducted: (i) a control treatment

340 with only seawater, (ii) a chemically dispersed oil treatment simulating, *in situ*, dispersant

341 application on an oil slick under mixing processes, (iii) dispersant alone in seawater as an

342 internal control of CD, (iv) a mechanically dispersed oil simulating only the effect of mixing

343 processes on the oil slick and (v) a water-soluble fraction of oil simulating contamination due

344 to an undispersed oil slick.

345 Given observations at oil spill sites (such as during the Braer oil spill, Lunel et al., 1995) and

346 the natural mixing processes in nearshore areas (e.g. waves), the presence of oil droplets in

347 the water column seems to be relevant when evaluating the toxicity of dispersant application

348 in nearshore areas. Thus, the experimental system was devised to maintain oil droplets in the

349 water column throughout the course of exposure.

350

351   4.1. Total petroleum hydrocarbons (TPH)

352

353   TPH concentrations vary from 46.4 to 14.9 mg/L for CD treatment and from 39.4 to 10.7

354   mg/L for MD treatment. The concentrations observed at T = 0 h are inferior to the nominal

355   concentrations (66.6 mg/L). This is probably due to the petroleum adherence to the

356   experimental system during the 24 h period of homogenisation (prior to the bioassays,

357   described in **2.1.5.**). The concentrations of TPH, measured in this experimental approach, are

358   consistent with those observed at oil spill sites. Indeed, Spooner (1970) observed 50 mg/L of

359   TPH after an oil spill in Tarut Bay (Saudi Arabia) due to a pipeline fracture. This observed

360   concentration was due to the natural dispersion of 16 000 t of light Arabian crude oil in

361   nearshore areas (less than 2 km from the shoreline). In the same way, Lunel (1995) observed

362   concentrations varying between 1 and 100 mg/L during the wreck of the Braer on the

363   Scotland coast. The cargo released 86 000 t of Gullfaks crude oil which were naturally

364   dispersed due to severe wind conditions (Force 6 to 10).

365   Braer oil spill shows that, in nearshore areas, meteorological conditions could induce

366   dispersion of the oil slick during a period of more than one week. However, at most oil spill

367   sites in offshore areas, a decrease in concentration is observed over a 2 to 5 h period (Lessard

368   and Demarco, 2000). Situated between these two scenarios, our experimental approach

369   showed a decrease in TPH concentration over a 48 h period. Our observations suggest that

370   this decrease is mainly due to petroleum adherence to the experimental system. This

371   phenomenon of adherence to the experimental system simulates the adherence to the substrate

372   observed in field studies (Baca et al., 2006). In this study, adhered petroleum represents

373   approximately the nominal concentration of the petroleum minus the concentration of

374   petroleum assessed in the water column. Even if adhered petroleum represents a relevant

375   proportion of the petroleum (in particular at T = 48 h), fish were not directly exposed to this

376   fraction of the petroleum since (i) pelagic fish species, such as golden grey mullets, should

377   only be exposed to petroleum present in the water column; (ii) in our study, most of the

378   adhered petroleum was present in the funnel, for which fish do not have access.

379

380   4.2. PAH biliary metabolites

381

382   The relative concentration of PAH biliary metabolites (evaluated through fixed wavelength

383   fluorescence analysis) has often been used as an exposure biomarker (Camus et al., 1998; Aas

384   et al., 2000; Jung et al., 2009). PAH are well studied since they are considered to be the most

385   toxic compounds of petroleum. In our study we measured the biliary-derived metabolites

386   corresponding to PAH (alkylated and parents) of three different weights (naphthalene: 128.2

387    $g.mol^{-1}$, pyrene: 202.3 $g.mol^{-1}$, benzo[a]pyrene: 252.3 $g.mol^{-1}$). The results showed a

388   significant increase in the three PAH metabolites following the CD treatment, when compared

389   to WSF. This result is in accordance with many studies (Perkins et al., 1973; Cohen and

390   Nugegoda, 2000; Ramachandran et al., 2004; Lin et al., 2009) since it shows that the

391   application of dispersant on an undispersed oil slick increases PAH exposure. The same is

392   true of the MD treatment, when compared to WSF: mechanical dispersion increased pyrene

393   and benzo[a]pyrene exposure (however no significant difference was observed for

394   naphthalene-derived metabolites). This increase in PAH exposure, due to the dispersion

395   (chemical or mechanical), suggests an increase of toxicity for tested organisms. Indeed, PAH

396   are considered as carcinogenic and mutagenic (Eisler, 1987). Moreover, studies revealed that

397   PAH induce histopathological effects (Stentiford et al., 2003 ; Ortiz-Delgado et al., 2007),

398   inflammatory responses (Stentiford et al., 2003), oxidative stress (Sun et al., 2006 ; Oliveira et

399   al., 2008) and alterations of DNA integrity (Oliveira et al., 2007 ; Maria et al., 2002) in teleost

400   fish.

401   With regards to the MD and CD treatment, our results show that the differences in the relative

402   concentration of the metabolites seem to be linked to PAH toxicity: the more toxic a PAH, the

403   lower the difference, in metabolite concentration, between the two treatments. Indeed,

404   naphthalene-derived metabolites (described as low toxicity PAH in Petry et al., 1996 and

405   Bosveld et al., 2002) showed a 40% increase with CD treatment (when compared to MD

406   treatment). Pyrene-derived metabolites showed a 13% increase. No significant difference was

407   observed for benzo[*a*]pyrene-derived metabolites, which is considered as a carcinogenic PAH

408   and induces reactive oxygen species (Lemaire-Gony and Lemaire, 1993).

409

410   4.3. Total glutathione content (GSH)

411

412   When compared to the WSF treatment, the CD treatment induced a significant decrease in

413   total glutathione content in the gills. Several hypotheses may explain the decrease in GSH

414   content, such as the conjugation of glutathione to PAH through the increase in GST activity as

415   observed in Yin et al. (2007) or the decrease in GSH synthesis due to contaminant exposure

416   as described in Canesi et al. (1999). Whatever the physiological mechanism implicated, this

417   study shows that dispersant application induced a depletion of glutathione, which is the first

418   line cellular defence involved in many detoxification processes (Maracine and Segner, 1998).

419   Thereby, the chemical dispersion of an oil slick decreases the potential of fish to cope with

420   contaminated environments.

421   On the contrary, when compared to the MD treatment, the CD treatment did not induce a

422   significant decrease in the total glutathione content in the gills, suggesting that, even when the

423   oil slick is mechanically dispersed (e.g. due to meteorological conditions), the application of

424   dispersant does not significantly decrease the potential of the organism to cope with its

425   environment.

426   Benzo[*a*]pyrene- and pyrene-derived metabolite concentrations were correlated with the total

427   glutathione content in the gills. However no correlation was found between naphthalene-

428   derived metabolites and total glutathione content. Taken together, these results suggest that

429   glutathione depletion arises due to exposure to heavy PAH whereas light PAH would not be

430   involved in the observed decrease in glutathione.

431   In Milinkovitch et al. (2011), a similar experimental approach was conducted with the same

432   exposure treatments as described in this study (C, CD, MD, WSF, D). The total glutathione

433   content in fish liver was evaluated and appeared to follow the same pattern as the total

434   glutathione content in gills (exposed in this study): CD treatment induced a significant

435   decrease of total glutathione when compared to control treatment; and no significant

436   difference was observed between CD and MD treatments. However, no significant difference

437   was observed concerning the liver total glutathione content between WSF and CD exposure

438   whereas, in the present study, when studying the fish gills, a significant difference was

439   observed between these both conditions. This finding shows that, evaluating dispersant

440   application toxicity, gill seems to be a more sensitive target organ than liver. This relevant

441   sensitivity of gills could be due to the fact that gills are target organs immediately in contact

442   with the external environment and thereby immediately in contact with pollutants presents in

443   the water column.

444

445   4.4. Oxidative stress

446

447   PAH, when incorporated by the organism, are bound to a cellular aryl hydrocarbon receptor

448   (AhR). This binding induces the formation of a complex, the aryl hydrocarbon receptor

449   nuclear translocator (ARNT), which is delocalized in the nucleus of the cell and bound to the

450   xenobiotic regulatory element (XRE). This phenomenon increases the transcription rate of the

451 P4501A cytochrome genes (CYP1A) and by the way increases the synthesis *de novo* of the

452 cytochrome P450 enzymes and the catalytic activity of these enzymes (Stegeman, 1987). This

453 increasing activity enhances the cellular production of reactive oxygen species (Livingstone,

454 2001), which is counteracted by the antioxidant response (especially through enzymatic

455 antioxidant activities). When the production of ROS overwhelms the antioxidant response,

456 free reactive oxygen species can interact deleteriously with cellular components. Lipid

457 peroxidation is a marker of this impairment.

458 Our results showed no modulation of lipid peroxidation, suggesting a lack of free radical

459 attack due to PAH exposure. Moreover, no antioxidant response was observed. The absence

460 of oxidative stress could be due to the composition of the fish food. Indeed, even if fish were

461 fed during four weeks with a fish food free of additives (also called synthetics) antioxidants,

462 natural antioxidants (such as vitamins A, C and E) are presents in the food composition. This

463 consummation of antioxidants could have prevented fish against oxidative stress.

464 Another explanation concerning this lack of significance could also be due to the fact that the

465 exposure period was too short to induce ROS production. Indeed, although some studies have

466 shown some effects of PAH following a short exposure period ($\leq$ 48 h, Sun et al., 2006;

467 Oliveira et al., 2008), many studies investigated the effects of contaminants on oxidative

468 stress by exposing animals to longer periods (Kopecka-Pilarczyk and Correia, 2009; Jung et

469 al., 2009; Narghang et al., 2009; Hannam et al., 2010).

470

471 **5. Conclusion**

472

473 With regards to gill glutathione content and the relative concentration of PAH biliary

474 metabolites, the results of this study firstly demonstrate that WSF exposure may be less toxic

475 than CD exposure. These results are in accordance with the TPH concentrations measured in

476   sea water, suggesting, in accordance with the literature (Perkins et al., 1973; Cohen and

477   Nugegoda, 2000; Ramachandran et al., 2004; Lin et al., 2009), that the transfer of

478   hydrocarbons (from the sea surface to the water column) due to dispersant application led to

479   an increase of toxicity. Extrapolated to field operations, results of this study mean that

480   containment and recovery, rather than chemical dispersion of the oil slick, must be conducted.

481   However, depending on technical facilities and meteorological conditions, it is not always

482   possible to contain the oil slick. In some oil spill situations (e.g. rough sea and low viscosity

483   petroleum), dispersant is the only appropriated response technique.

484   Since a minimum sea energy is required before a dispersant functions effectively (Merlin,

485   2005) and since nearshore areas are considered to be turbulent zones (due to waves, wind and

486   swell) it seemed important, in this study, to evaluate the toxicity of dispersant application

487   under a mixing process. Comparison of MD and CD showed a significant difference

488   concerning low toxicity PAH-derived metabolites (naphthalene and pyrene) - these results are

489   in accordance with the TPH concentrations measured in sea water -. However, no significant

490   difference was found for benzo[*a*]pyrene-derived metabolites, which are considered to be

491   carcinogenic and to induce reactive oxygen species. Moreover no significant difference was

492   found between the glutathione content following the CD and MD treatments. Taken together,

493   these results suggest (i) no increase of toxic compounds exposure due to dispersant

494   application and (ii) no increase of organism detoxification response. These results suggest

495   that, when an oil slick is naturally dispersed, the application of dispersant seems to not

496   increase its environmental toxicity. These results are in accordance with a similar previous

497   study (Milinkovitch et al., 2011).

498   However, several limits of this experimental approach compel us to be cautious in our

499   conclusions. Indeed, the experimental approach is available only for a given turbulent mixing

500   energy (the energy induced by the experimental system). Moreover, this experimental

501 approach only takes into account the toxicity to pelagic teleost fish while other components of

502 the ecosystem are also likely to be impaired by dispersant application. An experimental

503 approach considering the environmental conditions and other components of an ecosystem

504 (benthic and demersal species) would provide supplementary information. In this context,

505 further studies as part of the DISCOBIOL project will evaluate the impact of dispersed oil on

506 burrowing organisms, demersal organisms (such as oysters) and pelagic species (such as

507 golden grey mullet) within an enclosed ecosystem (mesocosm).

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520 **References**

521

522 Aas, E., Baussant, T., Balk, L., Liewenborg, B., Andersen, O.K., 2000. PAH metabolites in

523 bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil

524 exposure: a laboratory experiment with Atlantic cod. Aquat. Toxicol. 51, 241-258.

525

526   Adams, G.G., Klerks, P.L., Belanger, S.E., Dantin, D., 1999. The effect of the oil dispersant

527   omni-clean on the toxicity of fuel oil n° 2 in two bioassays with the sheepshead minnow

528   *Cyprinodon variegatus*. Chemosphere 39, 2141-2157.

529

530   Akerboom, T.P., Sies, H., 1981. Assay of glutathione, glutathione disulfide and glutathione

531   mixed disulfides in biological samples. Methods Enzymol. 77, 373-382.

532

533   Allen , J. I. & Moore, N. M., 2004. Environmental prognostics: Is the current use of

534   biomarkers appropriate for environmental risk evaluation? Mar. Environ. Res. 58, 227-232.

535

536

537   Almroth, B.C., Sturve, J., Stephensen, E., Holth, T.F., Förlin, L., 2008. Protein carbonyls and

538   antioxidant defenses in corkwing wrasse (*Symphodus melops*) from a heavy metal polluted

539   and a PAH polluted site. Mar. Environ. Res. 66, 271-277.

540

541   Amiard-Triquet, C. and Amiard, J. C., 2008. Les biomarqueurs dans l'évaluation de l'état

542   écologique des milieux aquatiques. Paris. pp. 372.

543

544

545   Avci, A., Kaçmaz, M., Durak, I., 2005. Peroxidation in muscle and liver tissues from fish in a

546   contaminated river due to a petroleum refinery industry. Ecotox. Environ. Saf. 60, 101-105.

547
548

549   Baca, B., Ward, G.A., Lane, C.H., Schuler, P.A., 2005. Net environmental benefit analysis

550   (NEBA) of dispersed oil on nearshore tropical ecosystems derived from International Oil Spill

551   Conference, Miami, Florida, USA, pp. 1-4.

552

553 Bosveld, A.T.C., De Bie, P.A.F., Van den Brink, N.W., Jongepier, H., Klomp, A.V., 2002. In

554 vitro EROD induction equivalency factors for the 10 PAH generally monitored in risk

555 assessment studies in The Netherlands. Chemosphere, 49, 75-83.

556

557 Bruslé, J., 1981. Food and feeding in grey mullets, in: Oren O.H. (ed.), Aquaculture of grey

558 mullets. Cambridge University Press, Cambridge, pp. 185-217.

559

560 Camus, L., Aas, E., Børseth, J.F., 1998. Ethoxyresorufin-O-deethylase activity and fixed

561 wavelength fluorescence detection of PAH metabolites in bile in turbot (*Scophthalmus*

562 *maximus* L.) exposed to a dispersed topped crude oil in a continuous flow system. Mar.

563 Environ. Res. 46, 29-32.

564

565 Canesi, L., Viarengo, A., Leonzio, C., Filippelli, M., Gallo, G., 1999. Heavy metals and

566 glutathione metabolism in mussel tissues. Aquat. Toxicol. 46, 67-76.

567

568 Chapman, H., Purnell, K., Law, R.J., Kirby, M.F., 2007. The use of chemical dispersants to

569 combat oil spills at sea: A review of practice and research needs in Europe. Mar. Pollut. Bull.

570 54, 827-838.

571

572 Churchill, P.F., Dudley, R.J., Churchill, S.A., 1995. Surfactant-enhanced bioremediation.

573 Waste Manag. 15, 371-377.

574

575 Cohen, A.M., Nugegoda, D., 2000. Toxicity of three oil spill remediation techniques to the

576 Australian Bass (*Macquaria novemaculeata*). Ecotoxicol. Environ. Saf. 47, 178-185.

577

578   Cormack, D., 1977. Oil pollution. Chem. Ind. 14, 605-608.

579

580   Deisseroth, A., Dounce, A.L., 1970. Catalase: physical and chemical properties, mechanisms

581   of catalysis, and physiological role. Physiol. Rev. 50, 319-375.

582

583   Eisler, R., 1987. Polycyclic aromatic hydrocarbon hazards to fish, wildlife, and invertebrates:

584   a synoptic review U.S. Fish and Wildlife Service Biological Report, 85, 1.11.

585

586   Fusey, P., Oudot, J., 1976. Comparaison de deux méthodes d'évaluation de la biodégradation

587   des hydrocarbures in vitro. Mater. U. Organ. 4, 241-251.

588

589   Gautier, D., Hussenot, J. (Eds.), 2005. Les mulets des mers d'Europe. Synthèse des

590   connaissances sur les bases biologiques et les techniques d'aquaculture. Ifremer, Paris.

591

592   George-Ares, A., Clark, J.R., 2000. Aquatic toxicity of two Corexit dispersants. Chemosphere

593   40, 897-906.

594

595   Gérard-Monnier, D., Erdelmeier, I., Régnard, K., Moze-Henry, N., Yadan, J., Chaudière, J.,

596   1998. Reactions of 1-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals.

597   Analytical applications to a colorimetric assay of lipid peroxidation. Chem. Res. Toxicol. 11,

598   1176-1183.

599

600   Halliwell, B. & Gutteridge, J. M. C., 1999. Free radicals in biology and medecine. Oxford

601   University Press, Oxford. pp. 192.

602

603 Hannam, M.L., Bamber, S.D., Galloway, T.S., John Moody, A., Jones, M.B., 2010. Effects of

604 the model PAH phenanthrene on immune function and oxidative stress in the haemolymph of

605 the temperate scallop *Pecten maximus*. Chemosphere 78, 779-784.

606

607 Jung, J.H., Yim, U.H., Han, G.M., Shim, W.J., 2009. Biochemical changes in rockfish,

608 *Sebastes schlegeli*, exposed to dispersed crude oil. Comparative Biochemistry and Physiology

609 Part C: Toxicology & Pharmacology 150, 218-223.

610

611 Kopecka-Pilarczyk, J., Correia, A.D., 2009. Biochemical response in gilthead seabream

612 (*Sparus aurata*) to in vivo exposure to a mix of selected PAH. Ecotoxicol. Environ. Saf. 72,

613 1296-1302.

614

615 Lemaire-Gony, S., Lemaire, P., 1993. Interactive effects of cadmium and benzo(a)pyrene on

616 cellular structure and biotransformation enzymes of the liver of the European eel *Anguilla*

617 *anguilla*. Aquat. Toxicol. 22, 145-160.

618

619 Lessard, R.R., DeMarco, G., 2000. The significance of oil spill dispersants. Spill Sci.

620 Technol. Bull. 6, 59-68.

621

622 Lin, C.Y., Anderson, B.S., Phillips, B.M., Peng, A.C., Clark, S., Voorhees, J., Wu, H.-D.I.,

623 Martin, M.J., McCall, J., Todd, C.R., Hsieh, F., Crane, D., Viant, M.R., Sowby, M.L.,

624 Tjeerdema, R.S., 2009. Characterization of the metabolic actions of crude versus dispersed oil

625 in salmon smolts via NMR-based metabolomics. Aquat. Toxicol. 95, 230-238.

626

627 Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and

628 oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656-666.

629

630 Lunel, T., 1995. The Braer oil spill: oil fate governed by dispersion. International Oil Spill

631 Conference, Long Beach, California, USA.

632

633 Maracine, M., Segner, H., 1998. Cytotoxicity of metals in isolated fish cells: Importance of

634 the cellular glutathione status. Comparative Biochemistry and Physiology Part A 120, 83-88.

635

636 Maria, V. L., Correia, A. C. & Santos, M. A., 2002. *Anguilla anguilla* L. biochemical and

637 genotoxic responses to Benzo[a]pyrene. Ecotoxicol. Environ. Saf., 53, 86-92.

638

639

640 Martinho, F., Leitao, R., Neto, J.M., Cabral, H.N., Marques, J.C., Pardal, M.A., 2007. The use

641 of nursery areas by juvenile fish in a temperate estuary, Portugal. Hydrobiologia 587, 281-

642 290.

643
644

645 McKeown, B.A., 1981. Long-term sublethal and short-term high dose effects of physically

646 and chemically dispersed oil on accumulation and clearance from various tissues of juvenile

647 coho salmon, *Onchorhynchus kitsutch*. Mar. Environ. Res. 1, 295-300.

648
649

650 Mendonça Duarte, R., Tomio Honda, R., Luis Val, A., 2010. Acute effects of chemically

651 dispersed crude oil on gill ion regulation, plasma ion levels and haematological parameters in

652 tambaqui (*Colossoma macropomum*). Aquat. Toxicol. 97, 134-141.

653

654   Merlin, F.X., 2005. Traitement aux dispersants des nappes de pétrole en mer. CEDRE

655   (CEntre de Documentation de Recherche et d'expérimentations sur les pollutions accidentelles

656   des eaux), Brest, France.

657

658   Milinkovitch, T., Ndiaye, A., Sanchez, W., Le Floch, S., Thomas-Guyon, H., 2011. Liver

659   antioxidant and plasma immune responses in juvenile Golden grey mullet (*Liza aurata*)

660   exposed to dispersed crude oil. Aquat. Toxicol. 101:155-154.

661

662   Milinkovitch, T., Kanan, R., Thomas-Guyon, H., Le Floch, S., Effects of dispersed oil

663   exposure on bioaccumulation of polycyclic aromatic hydrocarbons and mortality of juvenile

664   *Liza ramada*. Accepted for publication in Science of the total environment.

665

666   Mitchell, F.M., Holdway, D.A., 2000. The acute and chronic toxicity of the dispersants

667   Corexit 9527 and 9500, water accommodated fraction (WAF) of crude oil, and dispersant

668   enhanced WAF (DEWAF) to *Hydra viridissima* (green hydra). Water Res. 34, 343-348.

669

670   Nahrgang, J., Camus, L., Gonzalez, P., Goksøyr, A., Christiansen, J.S., Hop, H., 2009. PAH

671   biomarker responses in polar cod (*Boreogadus saida*) exposed to benzo(a)pyrene. Aquat.

672   Toxicol. 94, 309-319.

673

674   Oliveira, M., Pacheco, M., Santos, M. A., 2007. Cytochrome P4501A, genotoxic and stress

675   responses in Golden grey mullet (*Liza aurata*) following short-term exposure to

676   phenanthrene. Chemosphere, 66, 1284-1291.

677

678   Oliveira, M., Pacheco, M., Santos, M.A., 2008. Organ specific antioxidant responses in

679   golden grey mullet (*Liza aurata*) following a short-term exposure to phenanthrene. Sci. Total

680   Environ. 396, 70-78.

681

682   Ortiz-Delgado, J. B., Segner, H., Arellano, M. & Sarasquete, C., 2007. Histopathological

683   alterations, EROD activity, CYP1A protein and biliary metabolites in Gilthead seabream

684   *Sparus aurata* exposed to Benzo(a)pyrene. Histology and Histopathology, 22, 417-432.

685

686   Paglia, D.E., Valentine, W.N., 1967. Studies on the quantitative and qualitative

687   characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70, 158-169.

688

689   Perkins, R.A., Rhoton, S., Behr-Andres, C., 2005. Comparative marine toxicity testing: A

690   cold-water species and standard warm-water test species exposed to crude oil and dispersant.

691   Cold Regions Sc.Technol. 42, 226-236.

692

693   Petry, T., Schmidz, P., Slatter, C., 1996. The use of toxic equivalency factors in assessing

694   occupational and environmental health risk associated with exposure to airborne mixtures of

695   polycyclic aromatic hydrocarbons (PAH). Chemosphere, 4, 639-648.

696

697   Ramachandran, S.D., Hodson, P.V., Khan, C.W., Lee, K., 2004. Oil dispersant increases PAH

698   uptake by fish exposed to crude oil. Ecotoxicol. Environ. Saf. 59, 300-308.

699

700   Salar Amoli, H., Porgamb, A., Bashiri Sadr, Z. & Mohanazadeh, F., 2006. Analysis of metal

701   ions in crude oil by reversed-phase high performance liquid chromatography using short

702   column. J. Chrom. A, 1118, 82-84.

703

704 Sies, H., 1999. Glutathione and its role in cellular functions. Free Rad. Biol. Med. 27, 916-

705 921.

706

707 Spooner, M.F., 1970. Oil spill in Tarut Bay, Saudi Arabia. Mar. Pollut. Bull. 1, 166-167.

708

709 Stegeman, J.J., 1987. Cytochrome P450 isozymes and monoxygenase activity in aquatic

710 animals. Environ. Health Perspect. 71, 87-95.

711

712 Stentiford, G. D., Longshaw, M., Lyons, B. P., Jones, G., Green, M. & Feist, S. W., 2003.

713 Histopathological biomarkers in estuarine fish species for the assessment of biological effects

714 of contaminants. Mar. Environ. Res. 55, 137-159.

715

716 Sun, Y., Yu, H., Zhang, J., Yin, Y., Shi H., Wang, X., 2006. Bioaccumulation, depuration and

717 oxidative stress in fish (*Carassius auratus*) under phenanthrene exposure. Chemosphere 63,

718 1319-1327.

719

720 Tiehm, A., 1994. Degradation of polycyclic aromatic hydrocarbons in the presence of

721 synthetic surfactants. Appl. Environ. Microbiol. 60, 258-263.

722

723 van der Oost, R., Beyer, J. & Vermeulen, N. P. E., 2003. Fish bioaccumulation and

724 biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol., 13, 57-

725 149.

726

727 Wooliams, J.A., Wiener, G., Anderson., P.H., McMurray, C.H., 1983. Variation in the

728 activities of glutathione peroxidase and superoxide dismutase and in the concentration of

729 copper in the blood in various breed crosses of sheep. Research in Veterinary Science 34,
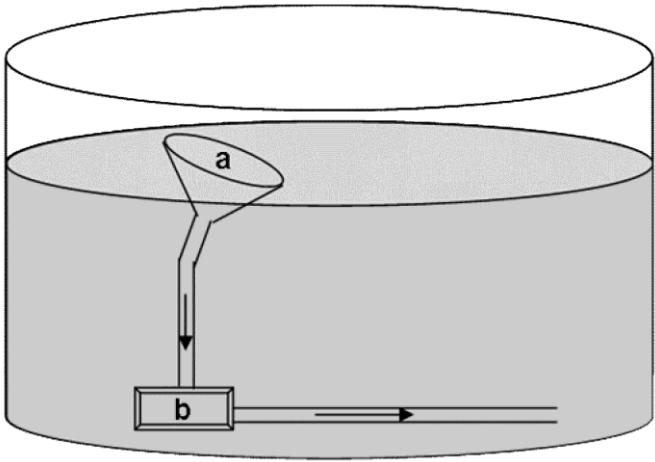
730 253-256

731

732 Yin, Y., Jia, H., Sun, Y., Yu, H., Wang, X., Wu, J., Xue, Y., 2007. Bioaccumulation and ROS

733 generation in liver of *Carassius auratus*, exposed to phenanthrene. Comparative Biochemistry

734 and Physiology Part C: Toxicology & Pharmacology 145, 288-293.

735
736 **Table 1:** TPH and dispersant nominal concentration in the five exposure media at the

737 beginning (T=0 h) and at the end of the exposure (T=48 h) to C (Control), CD (Chemically

738 Dispersed oil), MD (Mechanically Dispersed oil), WSF (Water Soluble Fraction of oil) and D

739 (Dispersant).

740 Values are expressed as mean ± standard error mean of both experimental replicates. n.d. =

741 not detected.

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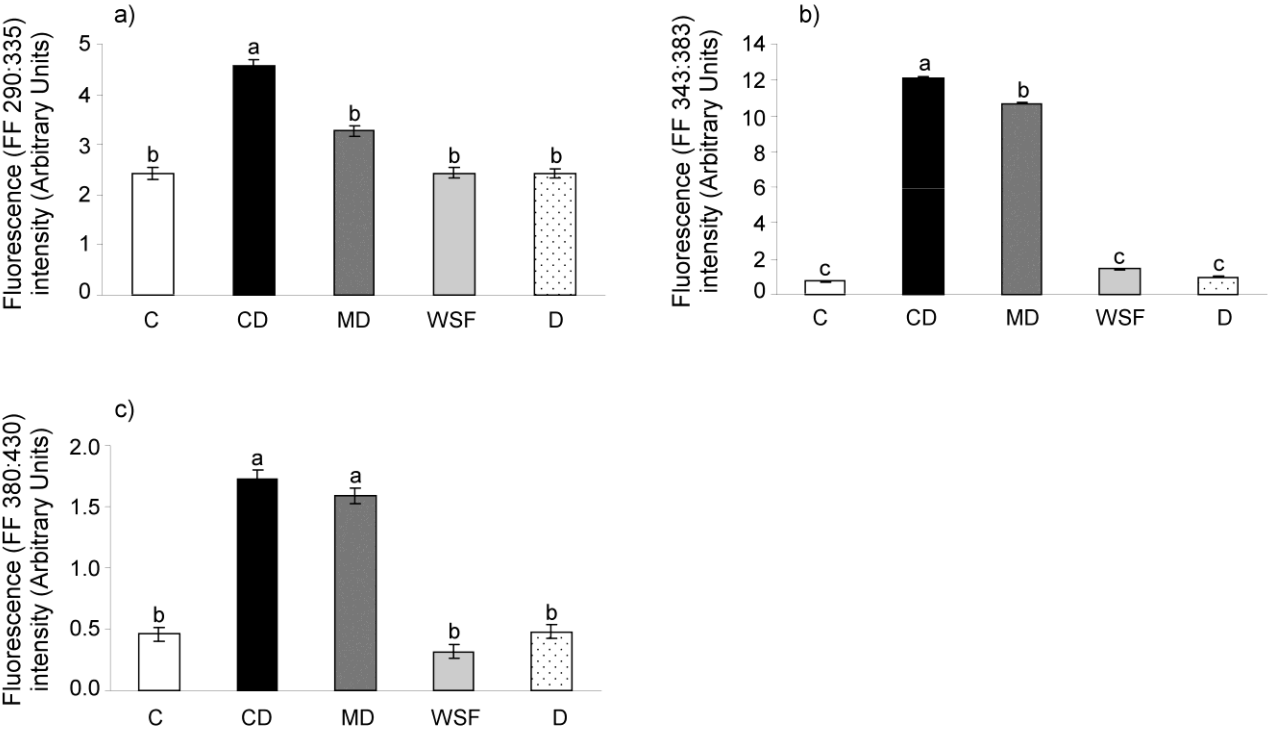|	$[TPH]_{T=0h}$ (mg/L)	$[TPH]_{T=48h}$ (mg/L)	$[Dispersant]_{nom.}$ (mg/L)
C	n.d.	n.d.	n.d.
CD	46.4	14.9	3.33
MD	39.2	10.7	n.d.
WSF	n.d.	n.d.	n.d.
D	n.d.	n.d.	3.33

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

749 300-l sea tank. → indicates the direction of seawater and/or contaminants through the

750 experimental system

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752

753   **Figure 2:** Concentration of biliary PAH metabolites measured by fixed wavelength

754   fluorescence (FF) levels after 48 h exposure to Control solution (C), Chemically Dispersed oil

755   solution (CD), Mechanically Dispersed oil solution (MD), Water Soluble Fraction (WSF)

756   solution and Dispersant solution (D) : (a) FF 290:335 (naphthalene type derived metabolites);

757   (b) FF 343:383 (pyrene derived type of metabolites); (c) FF 380:430 (benzo[*a*]pyrene type of

758   metabolites). Levels are expressed as fluorescence intensity. Values represent mean ±

759   standard error (n=10 per treatment). Different letters above bars indicate a significant

760   difference**,** where $P < 0.05$.
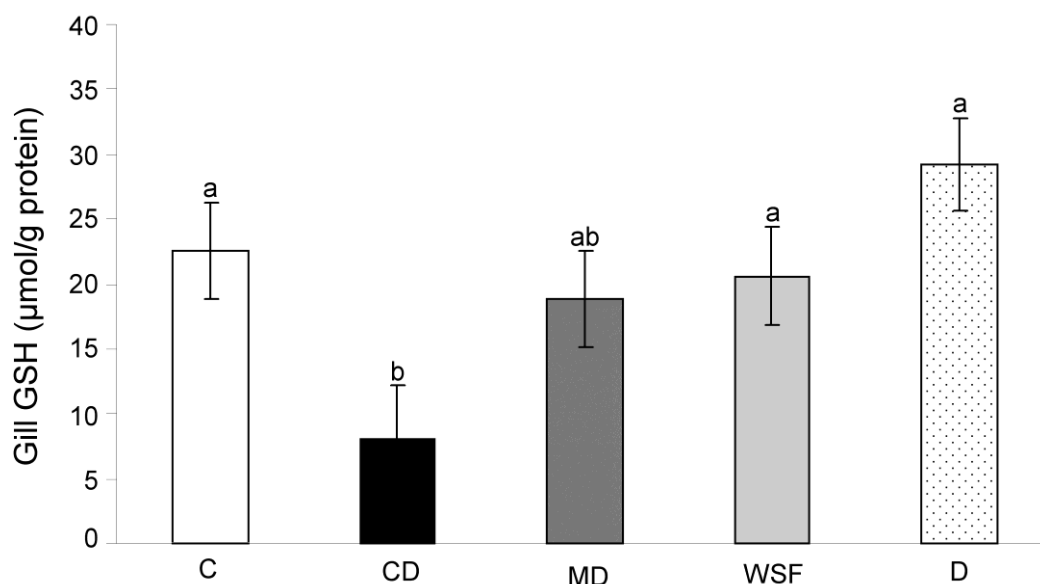
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762

763   **Figure 3**: Total glutathione (GSH) content in gills of *Liza aurata* after 48 h exposure to

764   Control solution (C), Chemically Dispersed oil solution (CD), Mechanically Dispersed oil

765   solution (MD), Water Soluble Fraction (WSF) solution and Dispersant solution (D). Values

766   represent mean ± standard error (n=10 per treatment). Different letters above bars indicate a

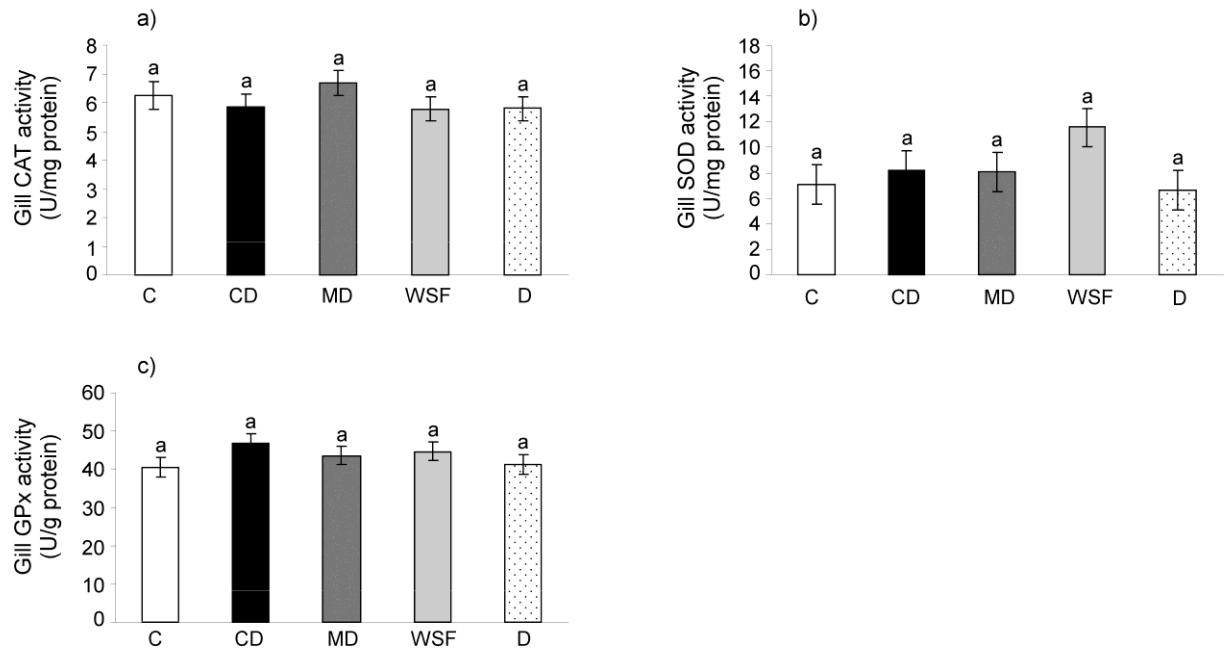767   significant difference**,** where $P < 0.05$.

768

Figure 4: a) Catalase (CAT) activity, b) Superoxide Dismutase (SOD) activity and c) Glutathione Peroxidase (GPx) activity in gills of *Liza aurata* after 48 h exposure to Control solution (C), Chemically Dispersed oil solution (CD), Mechanically Dispersed oil solution (MD), Water Soluble Fraction (WSF) solution and Dispersant solution (D). Values represent mean ± standard error (n=10 per treatment). Different letters above bars indicate a significant difference, where P < 0.05.
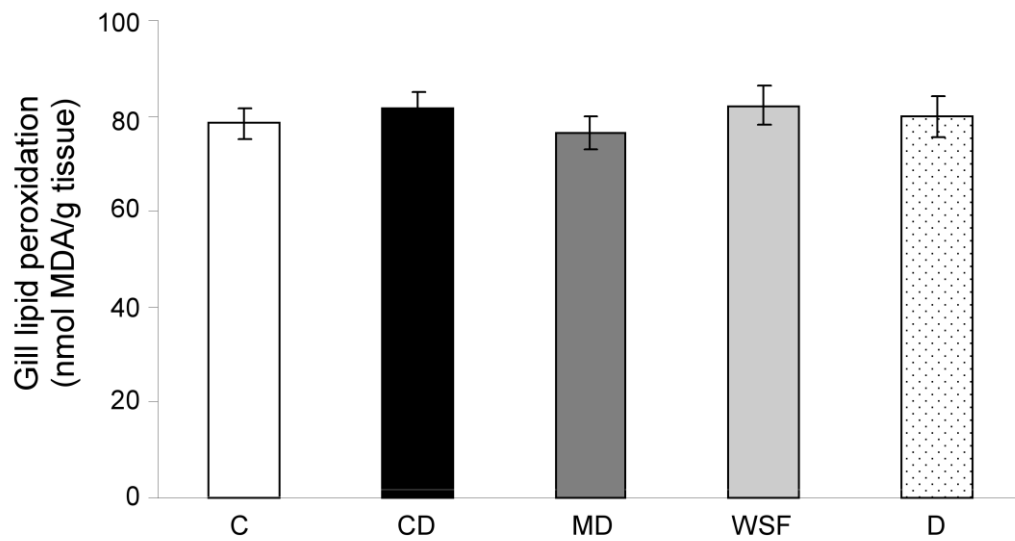
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Figure 5: Lipid peroxidation in gills of *Liza aurata* after 48 h exposure to Control solution (C), Chemically Dispersed oil solution (CD), Mechanically Dispersed oil solution (MD), Water Soluble Fraction (WSF) solution and Dispersant solution (D). Values represent mean ± standard error (n=10 per treatment). Different letters above bars indicate a significant difference, where $P < 0.05$.