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## Effects of dispersed oil exposure on the bioaccumulation of polycyclic aromatic hydrocarbons and the mortality of juvenile *Liza ramada*

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1 Effects of dispersed oil exposure on the bioaccumulation of polycyclic  
2 aromatic hydrocarbons and the mortality of juvenile *Liza ramada*

3

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23 **Abstract**

24

25 Dispersing an oil slick is considered to be an effective response to offshore oil spills.  
26 However, in nearshore areas, dispersant application is a controversial countermeasure:  
27 environmental benefits are counteracted by the toxicity of dispersant use. In our study, the  
28 actual toxicity of the dispersant response technique in the nearshore area was evaluated  
29 through an experimental approach using juvenile *Liza ramada*. Fish were contaminated via  
30 the water column (i) by chemically dispersed oil, simulating dispersant application, (ii) by  
31 dispersant, as an internal control of chemical dispersion, (iii) by mechanically dispersed oil,  
32 simulating only the effect of natural mixing processes, without dispersant application, and (iv)  
33 by the water soluble fraction of oil, simulating the toxicity of an oil slick before recovery.  
34 Bioconcentrations of polycyclic aromatic hydrocarbons (PAH) and mortality were evaluated,  
35 and related to both total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon  
36 (PAH) concentrations in seawater.

37 Fish exposed to chemically dispersed oil showed both a higher bioconcentration of PAH and a  
38 higher mortality than fish exposed to either the water soluble fraction of oil or the  
39 mechanically dispersed oil. These results suggest that (i) dispersion is a more toxic response  
40 technique than containment and recovery of the oil slick; (ii) in turbulent mixing areas,  
41 dispersant application increases the environmental risk for aquatic organisms living in the  
42 water column. Even if the experimental aspects of this study compel us to be cautious with  
43 our conclusions, responders could consider these results to establish a framework for  
44 dispersant use in nearshore areas.

45

46 **Keywords:** dispersant, chemically dispersed oil, toxicity, polycyclic aromatic hydrocarbons,  
47 bioaccumulation, oil droplets, nearshore areas, *Liza ramada*.

48

49 **1. Introduction**

50 In the last decades, increasing demand for petrochemicals has led to an increase in oil  
51 pollution in the sea. Many sources oil pollution, such as industrial wastewater, tanker  
52 accidents, and oil leaks from drilling operations (recently Deepwater Horizon) still  
53 contaminate the marine ecosystem. Even if oil spills do not represent the major source of  
54 pollution (one third of the petroleum hydrocarbons that enter the aquatic environment each  
55 year, UNEP/IOC/IAEA, 1992), the consequences of oil spills on local flora and fauna are  
56 disastrous (Dauvin, 1998; Claireaux et al., 2004; Cadiou et al., 2004). Secondary to  
57 mechanical containment and recovery of the oil slick, chemical dispersants can be used to  
58 reduce the environmental and economic impact of an oil spill. Chemical dispersants are  
59 composed of surface active agents (surfactants), which contain anionic and nonionic  
60 molecules that confer hydrophilic and hydrophobic properties, enabling lower interfacial  
61 tension between oil and water. This chemical process facilitates the formation of small, mixed  
62 oil-surfactant micelles, dispersed into the water column (Canevari, 1978). Thus, the  
63 application of chemical dispersants shows many advantages, by accelerating dilution of the  
64 oil slick (Lessard and Demarco, 2000) and consequently accelerating biodegradation of oil  
65 compounds (Thiem et al., 1994; Churchill, 1995). However, in nearshore areas, the  
66 advantages of dispersant use are counteracted by its toxicity: the low dilution potential of the  
67 oil slick in shallow water may expose ecologically sensitive ecosystems to relevant  
68 concentrations of petroleum. Therefore, in nearshore areas, the long-term net environmental  
69 benefits of dispersant application are counteracted by acute toxicity. A net environmental  
70 benefits analysis (NEBA, as conducted by Baca et al., 2005, on a mangrove ecosystem),  
71 which considers both the advantages and toxicity of dispersant use, is required in order to  
72 establish a comprehensive framework for dispersant use policies for nearshore areas.

73 In an attempt to do so, past studies have evaluated the acute toxicity of single dispersants  
74 (Perkins et al., 1973; Thompson and Wu, 1981; Law, 1995; Adams et al., 1999; George-Ares  
75 and Clark, 2000). More recent studies have taken into consideration the toxicity of the  
76 petroleum-dispersant interaction (Epstein et al., 2000; Long and Holdway, 2002; Lin et al.,  
77 2009) using chemical enhanced water accommodated fractions (CEWAF, Singer et al., 2000)  
78 as contamination solutions. These exposure solutions do not take into account most of the  
79 particulate oil formed during the dispersion of an oil slick.

80 However, the dispersion of oil provokes the presence of oil droplets, which have been  
81 suggested to be a determinant of toxicity (Ramachandran et al., 2004a and Brannon et al.,  
82 2006), and does so even more in nearshore areas, where natural dispersion (e.g. waves) can  
83 send the whole oil slick from the surface into the water column (as described during the Braer  
84 oil spill by Lunel et al., 1995). Thus, in order to simulate actual exposure to dispersed oil in  
85 nearshore areas, an experimental approach was designed. This approach, similar to  
86 Milinkovitch et al. (2011), considers the presence of oil droplets in the water column.

87 Experiments were conducted on juvenile, thin-lipped grey mullets (*Liza ramada*), because  
88 this teleost fish species is present in the Atlantic nearshore area during its early life stage  
89 (Gautier and Hussenot, 2005), and is consequently considered to be a target organism of  
90 anthropogenic contaminants (Bruslé, 1981). Moreover, this species is a key species of  
91 ecosystems since it plays a significant role in the global energy budgets of coastal  
92 environments, by transporting particulate organic matter from the salt marsh to the marine  
93 coastal waters (Lafaille et al., 1998). Four exposure conditions were tested on these  
94 organisms: (i) chemically dispersed oil solutions, simulating the application of dispersant  
95 when turbulent mixing processes (necessary for this response technique) are presents; (ii)  
96 single dispersant solutions as internal controls of chemically dispersed oil solutions; (iii) a  
97 mechanically dispersed oil solution, simulating the natural dispersion of an oil slick due to

98 mixing processes, but without dispersant application; (iv) a water soluble fraction of oil  
99 solution, simulating an undispersed oil slick before recovery, when the absence of turbulent  
100 mixing processes permit this response technique. Mortality was observed upon increasing  
101 exposure concentrations, yielding information on acute toxicity following dispersant  
102 application. Moreover, the concentration of 21 polycyclic aromatic hydrocarbons (PAH) was  
103 measured in both the sea water and fish muscles, since (i) PAH are considered to be a primary  
104 determinant of petroleum toxicity for aquatic organisms (Anderson et al., 1974) and (ii) their  
105 bioaccumulation in organisms is enhanced by dispersant application (Wolfe et al., 2001;  
106 Ramachandran et al., 2004b; Mielbrecht et al., 2005).

107

108

## 109 **2. Materials and methods**

110

### 111 **2.1 Materials and experimental organisms**

112

#### 113 **2.1.1. Experimental system**

114

115 The experimental system (**Figure 1**) used in this study was adapted from Blackman et al.  
116 (1978), and is composed of 12 experimental tanks (units) covered by a lid. Each tank is a 22 L  
117 cylinder fitted with a removable central column (77 mm in diameter), that houses a stainless  
118 steel shaft and a three-bladed propeller. The central cylinder has two sets of two apertures,  
119 located at the top and the bottom. The apertures are covered with a metallic mesh screen to  
120 exclude test animals from the propeller housing. The propeller (30 × 25 mm) is rotated (1000  
121 rounds per minute) to produce a small vortex within the central cylinder, drawing the  
122 exposure solutions in through the upper apertures and expelling them through the lower ones.

123 Even if residual oil is observed in the experimental system following exposure, the system is  
124 devised to maintain a mixture of oil-dispersant droplets throughout the water column. The  
125 system is a static water system, stored in a temperature controlled room (19 °C).  
126 Physicochemical parameters were measured during exposure (**Table 1**).

127

### 128 **2.1.2 Chemicals**

129

130 A Brut Arabian Light (BAL) oil was selected for this study and the composition of the oil was  
131 evaluated by CEDRE (CEntre de Documentation de Recherche et d'Expérimentations sur les  
132 pollutions accidentelles des eaux). The oil was found to contain 54% saturated hydrocarbons,  
133 36% aromatic hydrocarbons, and 10% polar compounds. Concentrations of 21 PAHs  
134 (including the 16 priority PAHs listed by US-EPA) in the Brut Arabian Light oil are presented  
135 in Table 2. Before performing the exposure studies, the oil was weathered by bubbling air  
136 through the petroleum in 3 m<sup>3</sup> tanks, for 8 days in open air, at a temperature of 12 to 16 °C.  
137 This aeration protocol results in a 7 % petroleum weight loss. This, corresponds to the  
138 petroleum weight loss occurring in 12 h on a 1 mm oil slick released at sea (personal  
139 communication, S. Le Floch). Using this weathered oil, our study simulates a 12 h period of  
140 petroleum ageing, i.e. the time it might take for responders to apply dispersant. The  
141 composition of the weathered test oil was 54% saturated hydrocarbons, 34% aromatic  
142 hydrocarbons and 12% polar compounds and its API (American Petroleum Institute) gravity  
143 was 33. Concentrations of 21 PAH (including the 16 US-EPA PAH) are presented in Table 2.  
144 Two formulations of dispersants (1 and 2), manufactured by Total Fluides and Innospech,  
145 were selected. Both were evaluated by CEDRE and were deemed effective enough  
146 (determined using the method, NF.T.90-345) for use in marine environments, non-toxic at  
147 concentrations recommended by the manufacturer (determined using a standard toxicity test:

148 method NF.T.90-349), and biodegradable. Dispersants 1 and 2 are composed of surfactants  
149 (surface active agents) and solvents. Because they are “third generation” dispersants, these  
150 surfactants are blends of anionic and non-ionic types (Fiocco and Lewis, 1999). The  
151 manufacturers state that the chemical compounds in their surfactants which represent a health  
152 risk are non-ionic surfactants (24 %) and anionic surfactants (between 12 and 24%) for  
153 dispersant 1; and saturated hydrocarbons with a flash point higher than 60 °C for dispersant 2.

154

### 155 **2.1.3 Experimental Organisms**

156

157 Juvenile, thin-lipped grey mullets (*Liza ramada*) were caught in Daoulas Bay (France) and  
158 acclimatised for 1 month in 300 L flow-through tanks ( $35 \pm 0.5$  ‰,  $19 \pm 0.2$  °C, with 12 h  
159 light:12 h dark photoperiods) prior to the bioassays. During acclimatisation, no mortality was  
160 observed and mullets were fed daily with fish food (Neosupra AL2 from Le Gouessant  
161 aquaculture). The fish were not fed 48 h prior to the bioassays, and throughout the exposure  
162 period. For each exposure condition, ten fish were weighed prior to the exposure (**Table 1**).

163

## 164 **2.2 Exposure Methods**

165

### 166 **2.2.1 Preparation of exposure media**

167

168 Stock solutions were prepared in 22 L glass beakers. All stock solutions were stirred for 24 h  
169 as described below. A Water Soluble Fraction of oil (WSF) stock solution was prepared as the  
170 Water Accommodated Fraction (WAF) recommended by CROSSERF, with the exception  
171 that the WAF preparation used in this study did not have a lid on the glass beaker in order to  
172 simulate the evaporation of light compounds which occur during oil slick confinement.



173 Practically, 95 g of BAL (Brut Arabian Light) oil was weighed and gently spread out over 20  
174 L seawater to simulate an oil slick. Then, the solution was stirred using a magnetic agitator  
175 (RCT basic IKA) using the low energy method (no vortexing) for a 24 h period. Only the  
176 liquid phase of the WSF of oil was used in the subsequent exposure studies. Chemically  
177 dispersed oil (CD1 and CD2) stock solutions, using dispersant 1 and 2, were prepared using  
178 20 L of seawater, 95 g BAL oil slick, and 5 g of dispersants 1 and 2 (following the  
179 manufacturers' recommended application petroleum:dispersant ratio of 20:1). Dispersant (D1  
180 and D2) stock solutions were prepared using 20 L of seawater and 5 g of dispersant. A  
181 mechanically dispersed oil stock solution (MD) was prepared using 20 L of seawater and 95 g  
182 BAL oil slick. CD1, CD2, D1, D2 and MD stock solutions were each stirred for 24 h using a  
183 propeller mixer (RW 16 Basic IKA), fitted with the same propeller as used in the  
184 experimental system. The propeller mixer speed was set higher than during exposure (1400  
185 rounds per minute instead of 1000 rounds per minute) in order to avoid the formation of an oil  
186 slick for the MD stock solution.

187

### 188 **2.2.2. Exposure conditions**

189

190 Following the 24 h period of exposure media preparation, each stock solution was diluted in  
191 seawater, which was previously placed in the experimental system tanks (described in **2.1.1.**).  
192 On the basis of one dilution per tank, 6 dilutions of each stock solution were made: 0%, 2.4%,  
193 12%, 18%, 24%, and 40%. The final volume of each dilution was 16 L. Two exposure  
194 conditions were tested simultaneously: CD1 and D1, followed by CD2 and D2, and then WSF  
195 of oil and MD, chronologically. Each group of 10 organisms was exposed to one stock  
196 solution dilution (16 L) for 24 h in one experimental tank. At the end of the 24 h exposure  
197 period, the animals in each tank were gently transferred to a 22 L glass tank with clean

198 seawater flow-through for 24 h, as recommended by Blackman et al. (1978). After 24 h, each  
199 tank was inspected and dead animals were counted. Animals were considered to be dead  
200 when no gill movement was visible and no response to a caudal pinch was observed.  
201 Surviving fish were euthanised using Eugenol (4-allyl-2-methoxyphenol). The whole axial  
202 muscle of each fish was removed and stored at -20°C for later assessment of polycyclic  
203 aromatic hydrocarbon (PAH) concentrations.

204

## 205 **2.3. Chemical analysis**

206

### 207 **2.3.1. Total petroleum hydrocarbon (TPH) seawater concentrations**

208

209 The TPH concentration, which is the sum of dissolved hydrocarbon concentrations plus the  
210 amount of oil droplets, was measured for all exposure media in each tank, at T = 0 h, and at  
211 the end of fish exposure (T = 24 h), using the mean of three replicated measurements for each  
212 time point. Each sample was removed using a Teflon straw, linked to a pipette filler (VWR),  
213 and stored in a 60 mL tinted glass bottle (VWR). The seawater samples were extracted with  
214 10 mL of Pestipur-quality dichloromethane (Carlo Erba Reactifs, SDS). After separation of  
215 the organic and aqueous phases, the seawater was extracted two additional times with the  
216 same volume of dichloromethane ( $2 \times 10$  mL). The combined extracts were dried on  
217 anhydrous sulphate and then analysed using a UV spectrophotometer (UV-Vis  
218 spectrophotometer, Unicam) at 390 nm, as described by Fusey and Oudot (1976). The  
219 detection limit of this method is dependent on the precision of the spectrophotometer, and  
220 results are not reliable for concentrations under 1 mg/L

221

### 222 **2.3.2. Polycyclic aromatic hydrocarbon (PAH) seawater concentrations**

223

224 PAH concentrations were assessed in each tank, at T = 0 h and following fish exposure (T =  
225 24 h), using the mean of two replicated measurements for each time point. After sampling, a  
226 24 h settling phase was used to separate oil droplets and particulate matter from the seawater.  
227 Then, 150 µL of a solution of 5 perdeuterated internal standards (Naphthalene d<sub>8</sub>, Biphenyl  
228 d<sub>10</sub>, Phenanthrene d<sub>10</sub>, Chrysene d<sub>12</sub>, and Benzo[a]pyrene d<sub>12</sub> at concentrations of 210, 110,  
229 210, 40 and 40 µg/mL, respectively in acetonitrile Sigma-Aldrich, France) were diluted in 10  
230 mL of absolute methanol (Sigma-Aldrich, France), and this volume of methanol was added to  
231 the liquid phase of the samples. PAH were extracted from the seawater using the stir bar  
232 sorptive extraction technique (SBSE, stir bar coated with PDMS, Gerstel), and analysed by  
233 thermal desorption coupled to a capillary gas chromatography–mass spectrometer (GC–MS).  
234 An HP7890 series II (Hewlett Packard, Palo Alto, CA, USA) GC was used, coupled with an  
235 HP5979 mass selective detector (MSD, Electronic Impact: 70eV, voltage: 2 000 V). Twenty-  
236 one PAH (alkylated and parents), including the 16 PAH listed by the US-EPA and 5  
237 additional PAH (benzo[*b*]thiophene, biphenyl, dibenzothiophene, benzo[*e*]pyrene, perylene),  
238 were quantified according to published procedures (Roy et al., 2005). Based on the detection  
239 limits of this method, accurate results at concentrations of 1ng/L were possible.

240

### 241 **2.3.3 Polycyclic aromatic hydrocarbon (PAH) concentrations in fish muscles and** 242 **bioaccumulation factor (BAF)**

243

244 The concentrations of 21 PAH (alkylated and parents) in the fish muscles were assessed. The  
245 21 PAH represent the 16 US-EPA PAH and 5 supplementary PAH (benzo[*b*]thiophene,  
246 biphenyl, dibenzothiophene, benzo[*e*]pyrene, perylene). PAH concentrations in the fish  
247 muscles were determined by GC–MS, using a procedure modified from Baumard et al.

248 (1997). Fish samples were pooled according to exposure treatment (one pool per tank).  
249 Although most pools contained some fish which survived the exposure experiments, pools  
250 were not analysed for exposures to 40 % stock solutions, because there were no surviving fish  
251 following CD1 and CD2 exposure.. The mean weight of the fish in the pools was  $6.1 \pm 0.7$  g.  
252 Prior to extraction, 150  $\mu$ L of a solution of 5 perdeuterated internal standards (Naphthalene  $d_8$ ,  
253 Biphenyl  $d_{10}$ , Phenanthrene  $d_{10}$ , Chrysene  $d_{12}$ , and Benzo[a]pyrene  $d_{12}$  at concentrations of  
254 210, 110, 210, 40 and 40  $\mu$ g/mL, respectively, in acetonitrile Sigma-Aldrich, France) and  
255 50 mL of an ethanolic solution of potassium hydroxide ( $2 \text{ mol L}^{-1}$ , Fisher Chemicals) were  
256 added to fish muscles in 250 mL flasks and placed for 3 h in a drying cupboard at  $60^\circ\text{C}$ .  
257 After alkaline digestion, 20 mL of demineralised water was added and samples were extracted  
258 with  $2 \times 20$  mL of pentane (Carlo Erba Reactifs, SDS). The resulting extract was then  
259 concentrated using a Turbo Vap 500 concentrator (Zyman, Hopkinton, MA, USA, at 880  
260 mbar and  $50^\circ\text{C}$ ) to 1 mL, purified on a silica column (5 g of silica, hydrocarbons were eluted  
261 with 50 mL of pentane/dichloromethane 80/20) and concentrated to 200  $\mu$ L for analysis.  
262 Aromatic compounds were analysed by GC–MS, with an approximate quantification limit of  
263  $5 \mu\text{g.kg}^{-1}$  of dry weight. PAH levels were quantified relative to the 5 perdeuterated internal  
264 standards introduced at the beginning of the sample preparation procedure (one per  
265 aromaticity class).  
266 Moreover, as described in Baussant et al. (2009), a bioaccumulation factor (BAF) was  
267 calculated using the ratio of the total PAH concentration in fish muscles divided by the total  
268 PAH concentration in seawater (**2.3.2.**).

269  
270  
271  
272

## 273 **2.4. Statistical analysis**

274

275 All correlations were tested using Spearman's correlation test (XL Stat 5.2) and the statistical  
276 significance of the results was ascertained at  $\alpha = 0.05$ . Differences between exposure  
277 conditions (CD1, CD2, MD, D1, D2, WSF) concerning seawater PAH concentrations, muscle  
278 PAH concentrations, and bioaccumulation factors were evaluated using the Quade test. For  
279 the Quade test procedure, exposure conditions were defined as treatment and % of stock  
280 solutions defined as blocks. Values obtained for each exposure conditions at several % of  
281 stock solutions were considered as repeated measurements. The Quade test was carried out  
282 using R statistical software and the statistical significance of the results was ascertained at  $\alpha =$   
283 0.05.

284

285

## 286 **3. Results**

287

288 Physicochemical parameters were stable and no difference was observed between exposure  
289 conditions (**Table 1**).

290

### 291 **3.1. Total petroleum hydrocarbon (TPH) seawater concentrations and fish mortality** 292 **(Tables 3a and 3b)**

293

294 No mortality was observed and the TPH concentration was zero for 0% stock solutions of all  
295 exposure media. Spearman's test revealed a correlation between TPH concentration (the mean  
296 of measurements at T = 0 h and at T = 24 h) and the percent dilution of stock solutions for  
297 CD1 and CD2 exposure ( $P < 0.05$ ), but no correlation was found for MD exposure ( $P =$

298 0.137). Because only soluble compounds are present in WSF of oil exposure media, the low  
299 TPH concentrations contained in this exposure media cannot be detected using  
300 spectrophotometry.

301 No mortality was observed following WSF of oil and MD exposure. For D1 exposure, 10 %  
302 mortality and 30 % mortality was observed for 18 and 40 % stock solution exposures,  
303 respectively. Approximately the same pattern was observed for D2 exposure: 10% mortality  
304 and 20 % mortality was observed for 12 and 40 % stock solution exposures, respectively.

305 For CD1 exposure, no mortality was observed following exposure to 0% and 2.4 % of the  
306 stock solution. Following exposure to 12, 18 and 24 % of the stock solution, 30 % mortality  
307 was observed. Thus, no mortality increase was observed following exposure between 12 and  
308 24 % of the CD1 stock solution, whereas our results show increased TPH concentrations.  
309 Following exposure to 40 % of the stock solution, 100 % mortality was observed.

310 For CD2 exposure, 0 % mortality was observed following exposure between 0 and 18 % of  
311 the stock solution. 10% mortality was observed following exposure to 24 % of the stock  
312 solution and 100 % mortality was observed following exposure to 40 % of the CD2 stock  
313 solution.

314

### 315 **3.2. Polycyclic aromatic hydrocarbon (PAH) seawater concentration**

316

317 No polycyclic aromatic hydrocarbons (PAH) were detected for 0% of the stock solutions for  
318 all exposure media.

319 A correlation between the sum of the concentrations of the 21 PAH (**Figure 2**) and the  
320 percent dilution of the stock solution was found for CD1, CD2 and WSF of oil exposures ( $P <$   
321  $0.05$ ), but not for MD exposure ( $P = 0.100$ ). The sum of the concentrations of the 21 PAH in  
322 seawater revealed significant differences between exposure conditions. First, PAH

323 concentrations were significantly higher following CD2 exposure than CD1 exposure.  
324 Moreover, our results reveal that exposure to chemically dispersed oil solutions (CD1 and  
325 CD2) is associated with higher concentrations of PAH than mechanically dispersed oil media.  
326 Finally, PAH concentrations following WSF of oil exposure were significantly lower than  
327 PAH concentrations measured following other exposure conditions (MD, CD1, CD2).

328

### 329 **3.3. Polycyclic aromatic hydrocarbon (PAH) concentrations in fish muscles and** 330 **bioaccumulation factor (BAF)**

331

332 Polycyclic aromatic hydrocarbon (PAH) concentrations in fish muscles (**Table 4**) were  
333 measured to be zero following exposure to 0% of the stock solutions for all exposure media.

334 Correlations were found between PAH concentrations in fish muscles and the percent stock  
335 solution dilution used for MD, WSF, CD1 and CD2 ( $P < 0.05$ ). PAH concentrations were  
336 significantly higher in chemically dispersed oil (CD1 and CD2) than in either mechanically  
337 dispersed oil (MD) or WSF of oil. Even though the PAH concentrations appeared to be much  
338 higher following mechanically dispersed oil exposure than WSF of oil exposure, statistical  
339 analysis did not reveal any significant difference ( $P$ -value = 0.115).

340 No correlation was observed between BAF and the percent stock solution dilution, for all  
341 exposure media (**Table 5**). BAF was found to be significantly higher following exposure to  
342 chemically dispersed crude oil (CD1 and CD2) than WSF of oil exposure. Although BAF  
343 levels appeared to be higher following MD exposure than following WSF of oil exposure, no  
344 significant difference was found ( $P = 0.064$ ). No significant difference was found between  
345 MD and CD1 exposure ( $P = 0.265$ ). The same is true of MD and CD2 exposure ( $P = 0.701$ ).

346

347

348 **4. Discussion**

349

350 The aim of this study was to evaluate the toxicity due to dispersant application in nearshore  
351 areas as an oil spill response technique. Using an experimental approach, this study took into  
352 account the turbulent mixing processes inherent in nearshore waters, and also accounted for  
353 the presence of oil droplets resulting from oil slick dispersion. Four exposure conditions were  
354 tested: (i) chemically dispersed oil solutions, simulating the application of dispersant when  
355 turbulent mixing processes permit this response technique; (ii) dispersant alone (D1 and D2)  
356 in seawater, as internal controls of chemically dispersed oil solutions; (iii) a mechanically  
357 dispersed oil solution, simulating the natural dispersion of an oil slick due to mixing processes  
358 but without dispersant application; (iv) a water soluble fraction of oil, simulating an  
359 undispersed and untreated oil slick before recovery, when calm weather conditions permit this  
360 response technique.

361 For 0% dilutions of the stock solution of all treatments, mortality did not occur and  
362 contaminants were not detected in seawater or fish tissues. These results, coupled with the  
363 stability of physicochemical parameters (T °C, pH, dissolved oxygen, salinity) validate the  
364 experimental procedure used in this study.

365

366 **4.1. Mortality, total petroleum hydrocarbon (TPH), and polycyclic aromatic**  
367 **hydrocarbon (PAH) concentrations in seawater**

368

369 The TPH concentration decrease observed in this study between T = 0 h and T = 24 h, is in  
370 agreement with field operation measurements, although the drastic decrease observed in  
371 literature (Cormack, 1977; Lessard and Demarco, 2000) was slighter less in our experiment.  
372 Although the experimental system used for this study attempts to simulate natural dispersion



373 at nearshore areas, we admit that the evolution of TPH concentration depends on the turbulent  
374 mixing energy of the experimental system.

375 Correlations between the percentage stock solution used for exposure and total petroleum  
376 hydrocarbon concentrations (mean of measurements at T = 0h and at T = 24 h) were observed  
377 following CD1 and CD2 exposure, whereas no correlation was found following MD  
378 exposure. Indeed, following CD1 and CD2 exposure, TPH concentrations increase linearly as  
379 a function of the percentage stock solution used for exposure, whereas TPH concentrations for  
380 MD exposure did not increase between 12% of the stock solution and 40% of the stock  
381 solution. Extrapolated to oil spill response techniques, this result shows that, for a given sea  
382 energy (in this study, the experimental system energy), a threshold water column  
383 concentration cannot be exceeded if the oil slick is dispersed mechanically, whereas this  
384 threshold can be exceeded if the oil slick is dispersed chemically.

385 In parallel, an oil slick was observed following exposure to 12, 18, 24, and 40% MD stock  
386 solution dilutions, whereas no oil slick was observed following CD1 and CD2 exposure. Thus  
387 we can hypothesise that, following MD exposure, increasing the petroleum quantity led to an  
388 increase in oil slick thickness instead of an increase in water column TPH concentration. The  
389 formation of an oil slick during mechanical dispersion, but not during chemical dispersion is  
390 in agreement with the mode of action of chemical dispersants and with observations made at  
391 oil spill sites. Lewis and Daling (2001) gave a complete explanation of this phenomenon:  
392 when turbulent mixing energy permits natural dispersion of the oil slick, oil droplets are large  
393 (from 0.4 to several mm in diameter) and rise quickly back to the sea surface, where they  
394 coalesce and reform the oil slick. In contrast, chemical dispersant application mediates the  
395 formation of smaller oil droplets (10 to 50  $\mu\text{m}$ ), which have a low rise velocity and are  
396 disseminated, for instance by water currents, before they can reform an oil slick.

397 Regarding to fish mortality, chemically dispersed oil would be more toxic than an untreated  
398 and undispersed oil slick (WSF). Indeed, no mortality was found for WSF of oil exposure  
399 whereas 100 % fish mortality was observed following exposure to 40% dilutions of CD1 and  
400 CD2 exposure. Moreover, mortality due to chemical dispersion of oil (CD1 and CD2) were  
401 observed at lower concentrations: at 12 % of the CD1 stock solution and at 24 % of the CD2  
402 stock solution. Note that mortality did not increase between 12 % and 24 % of the CD2 stock  
403 solution, whereas actual TPH concentrations increased. This phenomenon is likely due to  
404 contamination resistance variability between fish. For example, it is possible that some fish  
405 exposed to 24 % of the CD2 stock solution were more resistant to hydrocarbon contamination  
406 than other fish exposed to 12 % of the CD2 stock solution. This would result in the same  
407 mortality percentage for both groups of fish, even if the exposure concentrations were  
408 different. In total, these results are in agreement with many studies (Long and Holdway, 2002;  
409 Pollino and Holdway, 2002; Lin et al., 2009) and suggest, as it was previously proposed by  
410 Cohen and Nugegoda (2000), that when meteorological conditions permit it, recovery and  
411 containment of the oil slick should be conducted since it is a less toxic response technique  
412 than the application of chemical dispersants. Similarly, chemically dispersed oil seems to be  
413 more toxic than mechanically dispersed oil, because no mortality was observed after fish were  
414 exposed to this condition. This finding suggests that, when the oil slick is under natural  
415 mixing processes, the application of chemical dispersant increases the toxicity of the  
416 petroleum, probably by increasing the amount of total petroleum hydrocarbons in the water  
417 column (as described above). Moreover, our study results suggest that the toxicity of  
418 dispersants (D1 and D2) seems to be too low to be the major determinant of CD1 and CD2  
419 exposure toxicity. This result is in accordance with Otitoluju (2005), who showed that the  
420 toxicity of dispersant application is not due to dispersant toxicity alone, but rather to the  
421 synergistic toxicity of dispersant and petroleum.

422 With respect to seawater concentrations of the 21 PAH compounds tested, our results show  
423 that PAH concentrations were significantly higher following CD1 and CD2 exposure than  
424 WSF of oil exposure. This result is in line with the mode of action of chemical dispersants,  
425 which increase the total surface area for the partitioning of PAH from oil to water by  
426 increasing the number of droplets and decreasing their size. Mechanical dispersion also  
427 increases the number of droplets in the water column, and, logically, based on our results, this  
428 phenomenon led to an increase in PAH concentrations (comparing WSF of oil exposure and  
429 MD exposure).

430 Our study results demonstrate that PAH concentrations are higher in chemically dispersed oil  
431 than in mechanically dispersed oil. This result could also be due to an increase in surface area  
432 available for PAH partitioning, since part of the petroleum is taking part in an oil slick  
433 (instead of droplets) in mechanically dispersed oil.

434 Moreover, our results indicate that, through comparison of both chemical dispersants (CD1  
435 and CD2), dispersant 2 induces higher PAH concentrations in seawater than dispersant 1.  
436 However, TPH concentrations in the water column do not seem to be different between both  
437 dispersed oil solutions. It is therefore possible to hypothesise that dispersant 1 retards the  
438 PAH partitioning from oil droplets to water.

439 In our study, the sum of 21 PAH concentrations in seawater were never higher than 308 µg/L  
440 (following exposure to 40 % of the CD2 stock solution). In a study by Maria et al. (2002),  
441 conducted on *Anguilla anguilla*, the exposure period was 9 times longer (216 h) than in our  
442 study, and the concentration of benzo[*a*]pyrene -considered to be the most toxic of the 21  
443 PAH (Eisler, 1987)- was measured to be 2 times higher (680 µg/L) than the sum of 21 PAH  
444 concentrations measured in our study. Nevertheless, in this last study, mortality was not  
445 observed. Comparison of these two studies suggests that, in our study, PAH concentration  
446 was not sufficient to induce mortality. Thus, PAH were not the only determinant of acute

447 toxicity. In fact, acute toxicity could be due to other petroleum compounds (such as saturated  
448 hydrocarbons), or the presence of oil droplets (Brannon et al., 2006). Therefore, as stated in  
449 the introduction of this article, the presence of total petroleum hydrocarbons and/or droplets in  
450 the water column seems to be very important for evaluation of the toxicity of chemically  
451 dispersed oil.

452

#### 453 **4.2. Bioaccumulation factor (BAF), PAH concentrations in fish muscles**

454

455 PAH compounds are considered to be mutagenic and carcinogenic (Ohe et al., 2004) and,  
456 consequently, their bioaccumulation is relevant and interesting because it provides  
457 information about long-term toxicity (Ramachandran et al., 2004a). In the present study, PAH  
458 bioconcentrations were measured following a 24 h period in clean seawater. There is evidence  
459 that biological detoxification processes (such as the induction of EROD activity described in  
460 Camus et al., 1998) can be induced during this period. However, because the detoxification  
461 period was the same for all fish, comparison of results between exposure conditions is  
462 reliable.

463 In a comparison of chemically dispersed oil and the water soluble fraction of oil, our results  
464 show that dispersion of the oil slick led to an increase in PAH concentrations in seawater  
465 (previously discussed in section 4.1. above). Moreover, our results show that PAH  
466 bioaccumulation (via BAF calculation) is higher following CD1 and CD2 exposure conditions  
467 than WSF of oil exposure conditions. This phenomenon could be due to other contaminants  
468 (such as saturated hydrocarbons or the chemical dispersant), which can induced functional  
469 morphology alterations in the gills (as described in Rosety-Rodriguez et al., 2002). This  
470 alteration would have induced a decrease of the selective permeability of gills and by the way  
471 an increase of PAH bioaccumulation.

472 Taken together, these results show that dispersant application increases PAH partitioning  
473 from oil to water, and moreover, increases PAH bioaccumulation (at the seawater–organism  
474 interface). As a result, PAH concentrations were found to be higher in the muscles of fish  
475 exposed to chemically dispersed oil than in the muscles of fish exposed to the water soluble  
476 fraction of oil. These results are in agreement with many studies that have revealed an  
477 increase in PAH uptake due to dispersant application (Wolfe et al., 2001; Mielbrecht et al.,  
478 2005).

479 Similarly, PAH concentration measurements in seawater were found to be higher following  
480 MD exposure than WSF of oil exposure. Moreover, these results show that PAH  
481 bioaccumulation seems to be higher following MD exposure than WSF of oil exposure ( $P =$   
482  $0.064$ ). Together, these results suggest that mechanical dispersion seems to increase PAH  
483 partitioning from oil to water, and, in addition, increase PAH bioaccumulation (at the  
484 seawater–organism interface). As a result, PAH concentrations seem to be higher in the  
485 muscles of fish exposed to mechanically dispersed oil (MD) than in the muscles of fish  
486 exposed to the water soluble fraction of oil ( $P$  value =  $0.115$ ).

487 By comparing chemically and mechanically dispersed crude oil, we have shown that PAH  
488 concentrations in seawater were higher following chemically dispersed oil exposure (either  
489 CD1 or CD2) than following MD exposure. PAH bioaccumulation (BAF) was not different  
490 following these two exposures whereas PAH concentrations were significantly higher in the  
491 muscles of fish exposed to chemically dispersed oil than in the muscles of fish exposed to  
492 mechanically dispersed oil. These results suggest that, following chemical dispersant  
493 application, PAH partitioning from oil to water is the main factor inducing the observed  
494 increase in PAH bioconcentrations.

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496

497 **5. Conclusion**

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499 By comparing chemically dispersed oil exposure and water soluble fraction of oil exposure,  
500 our results demonstrate that chemical dispersion is more toxic than an untreated and  
501 undispersed oil slick, both in terms of acute toxicity (i.e. mortality observations) and chronic  
502 toxicity (increased PAH bioconcentration in fish muscles). The higher toxicity found for  
503 chemically dispersed oil solutions is probably due to the presence of oil droplets and the  
504 resulting increase in the concentration of PAH in the water column. Based on these results,  
505 responders should consider the increased toxicity due to chemical dispersion before using this  
506 response technique. For instance, when an oil spill site is ecologically sensitive, oil  
507 dispersion, by applying dispersants and inducing mixing processes (e.g. using a boat propeller  
508 as recommended in Merlin et al., 2005), would not be appropriate. In this case, oil slick  
509 containment and recovery should be considered if technical facilities and meteorological  
510 conditions permit.

511 Our comparison of chemically and mechanically dispersed oil exposure effects has yielded  
512 information on dispersant application toxicity when turbulent mixing processes are present in  
513 nearshore areas. Under these conditions, oil slick containment and recovery is impossible,  
514 because of natural dispersion of the slick. Our results show that dispersant application  
515 increases both fish mortality and PAH bioconcentrations (by increasing the amount of  
516 dissolved PAH in the water column). These results suggest that, when the oil slick is under  
517 natural mixing processes, such as waves, the application of dispersant increases the  
518 environmental risk for aquatic organisms living in the water column.

519 However, these results were obtained at an experimental given mixing energy and  
520 concentrations used were significantly higher than those normally encountered during oil  
521 spills (Cormack, 1977; Lunel et al., 1995). These limitations of our study compel us to be

522 cautious in our conclusion. Nevertheless, responders must take into account these results and  
523 also need more information on potentially sublethal effects, to better evaluate the long-term  
524 toxicity of dispersant application. Because of this, our study is part of an on-going project  
525 (DISCOBIOL project: DISpersant and response techniques for COastal areas; BIOLogical  
526 assessment and contributions to the regulation). Considering both the toxicity of dispersant  
527 application and its advantages, this project aims to obtain information about the ecological  
528 effects of dispersant application in nearshore areas.

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530

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532

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540

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668 **Table 1:** Fish weight (Values represent mean  $\pm$  standard error mean; n = 10 per treatment)  
 669 and physicochemicals parameters measured during organism exposure (Values represent  
 670 mean  $\pm$  standard error mean of 6 tanks measurement at T = 0 h and at T = 24 h)

Parameters	MD	CD1	CD2	WSF	D1	D2
Temperature (°C)	19.1 $\pm$ 0.1	18.8 $\pm$ 0.1	18.6 $\pm$ 0.2	19.1 $\pm$ 0.1	18.9 $\pm$ 0.2	18.6 $\pm$ 0.2
pH	7.96 $\pm$ 0.03	7.92 $\pm$ 0.04	7.90 $\pm$ 0.03	8.00 $\pm$ 0.01	7.98 $\pm$ 0.02	7.93 $\pm$ 0.02
Dissolved oxygen (% AS)	95.2 $\pm$ 0.8	92.1 $\pm$ 0.6	94.6 $\pm$ 1.1	95.2 $\pm$ 0.8	93.8 $\pm$ 0.6	96.8 $\pm$ 0.6
Salinity (‰)	35.1 $\pm$ 0.1	35.2 $\pm$ 0.1	35.2 $\pm$ 0.1	35.1 $\pm$ 0.1	35.1 $\pm$ 0.1	35.2 $\pm$ 0.1
Fish weight (g)	1.74 $\pm$ 0.11	1.60 $\pm$ 0.12	1.65 $\pm$ 0.09	1.71 $\pm$ 0.12	1.78 $\pm$ 0.11	1.87 $\pm$ 0.14

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674 **Table 2:** Concentration of 21 PAHs (alkylated and parents) in the Brut Arabian Light (BAL)  
 675 and in the weathered Brut Arabian Light. The 21 PAHs represent the 16 US-EPA PAHs and  
 676 five supplementary PAHs (benzo[*b*]thiophene, biphenyl, dibenzothiophene, benzo[*e*]pyrene,  
 677 perylene). n.d. = not detected.

	Molecular weight (g/mol)	Concentration in BAL ( $\mu$ g/g of petroleum)	Concentration in weathered BAL ( $\mu$ g/g of petroleum)
Naphtalene	128.2	222	211
C1-Naphtalene	143.2	955	854
C2-Naphtalene	158.2	2 099	1 819
C3-Naphtalene	173.2	2 084	1 796

C4-Naphtalene	188.2	1480	1317
Benzo[ <i>b</i> ]thiophene	134.2	5	5
C1-benzo[ <i>b</i> ]thiophene	149.2	63	22
C2-benzo[ <i>b</i> ]thiophene	164.2	298	292
C3-benzo[ <i>b</i> ]thiophene	179.2	681	1 030
C4-benzo[ <i>b</i> ]thiophene	209.2	606	537
Acenaphtylene	152.2	30	25
Biphenyl	154.2	15	14
Acenaphtene	154.2	4	3
Fluorene	166.2	45	39
C1-Fluorenes	181.2	132	116
C2-Fluorenes	196.2	269	230
C3-Fluorenes	211.2	304	261
Phenanthrene	178.2	112	95
Anthracene	178.2	112	95
C1-phenanthrenes/anthracenes	193.2	396	335
C2-phenanthrenes/anthracenes	208.2	603	498
C3-phenanthrenes/anthracenes	223.2	493	416
C4-phenanthrenes/anthracenes	238.2	318	273
Dibenzothiophene	184.3	373	330
C1-dibenzothiophenes	199.3	1115	987
C2-dibenzothiophenes	214.3	2021	1759
C3-dibenzothiophenes	229.3	1764	1546
C4-dibenzothiophenes	244.3	1040	936
Fluoranthene	202.3	7	6

Pyrene	202.3	11	9
C1-fluoranthenes/pyrenes	217.3	62	51
C2-fluoranthenes/pyrenes	232.3	137	119
C3-fluoranthenes/pyrenes	247.3	222	191
Benzo[ <i>a</i> ]anthracene	228.3	19	16
Chrysene	228.3	18	15
C1-chrysenes	243.3	37	29
C2-chrysenes	258.3	57	45
C3-chrysenes	273.3	84	88
Benzo[ <i>b+k</i> ]fluoranthene	252.3	3	3
Benzo[ <i>e</i> ]pyrene	252.3	2	2
Benzo[ <i>a</i> ]pyrene	252.3	11	9
Perylene	252.3	3	7
Benzo( <i>g,h,i</i> )perylene	276.3	2	2
Indeno( <i>1,2,3-cd</i> )pyrene	276.3	n.d.	n.d.
Dibenz( <i>a,h</i> )anthracene	278.4	n.d.	1

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686 **Table 3a:** Mortality and Total petroleum hydrocarbon (TPH) concentration (values represent  
 687 the concentration at T<sub>0h</sub>-T<sub>24h</sub>) measured for each % of stock solution, for MD (mechanically  
 688 dispersed oil), CD1 (chemically dispersed oil using dispersant 1), CD2 (chemically dispersed  
 689 oil using dispersant 2). n.d. = not detected.

	MD		CD1		CD2	
% of stock solution	[TPH] (mg/L)	Mortality (%)	[TPH] (mg/L)	Mortality (%)	[TPH] (mg/L)	Mortality (%)
0	n. d.	0	n. d.	0	n. d.	0
2.4	67-15	0	55-35	0	111-64	0
12	281-228	0	491-398	30	616-548	0
18	173-158	0	873-605	30	777-625	0
24	383-340	0	1223-1096	30	1203-1055	10
40	374-122	0	1606-1457	100	1641-1438	100

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701 **Table 3b:** Mortality and contaminants concentration (TPH for WSF exposure and Dispersant  
 702 nominal concentration for D1 and D2 exposure). n.d. = not detected.

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	WSF		D1		D2	
% of stock solution	[TPH] (mg/L)	Mortality (%)	[Disp] (mg/L)	Mortality (%)	[Disp] (mg/L)	Mortality (%)
0	n. d.	0	0	0	0	0
2.4	n. d.	0	6	0	6	0
12	n. d.	0	30	0	30	10
18	n. d.	0	45	10	45	0
24	n. d.	0	60	0	60	0
40	n. d.	0	100	30	100	20

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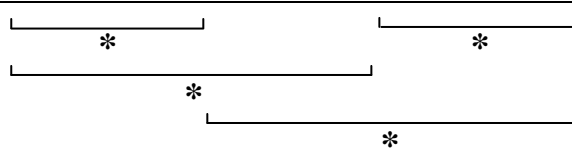
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717 **Table 4:** Concentration of the sum of 21 PAHs (alkylated and parents) in fish muscles ( $\mu\text{g/g}$ )  
 718 for CD1 (Chemically Dispersed oil using dispersant 1), CD2 (Chemically Dispersed oil using  
 719 dispersant 2), MD (Mechanically Dispersed oil) and WSF (Water Soluble Fraction of oil)  
 720 exposures. Respecting Quade test procedures, values obtained for each exposure conditions at  
 721 several % of stock solution are considered as repeated measure. \*indicates significant  
 722 differences ( $P < 0.05$ ) of concentrations ([sum of 21 PAHs]) between exposure conditions.  
 723 n.d. = not detected.

% of stock solution	MD	CD1	CD2	WSF
0	n. d.	n. d.	n. d.	n. d.
2.4	2.27	6.30	5.70	0.08
12	3.34	10.23	8.13	0.09
18	4.24	11.43	17.00	0.14
24	9.80	12.10	11.25	0.12

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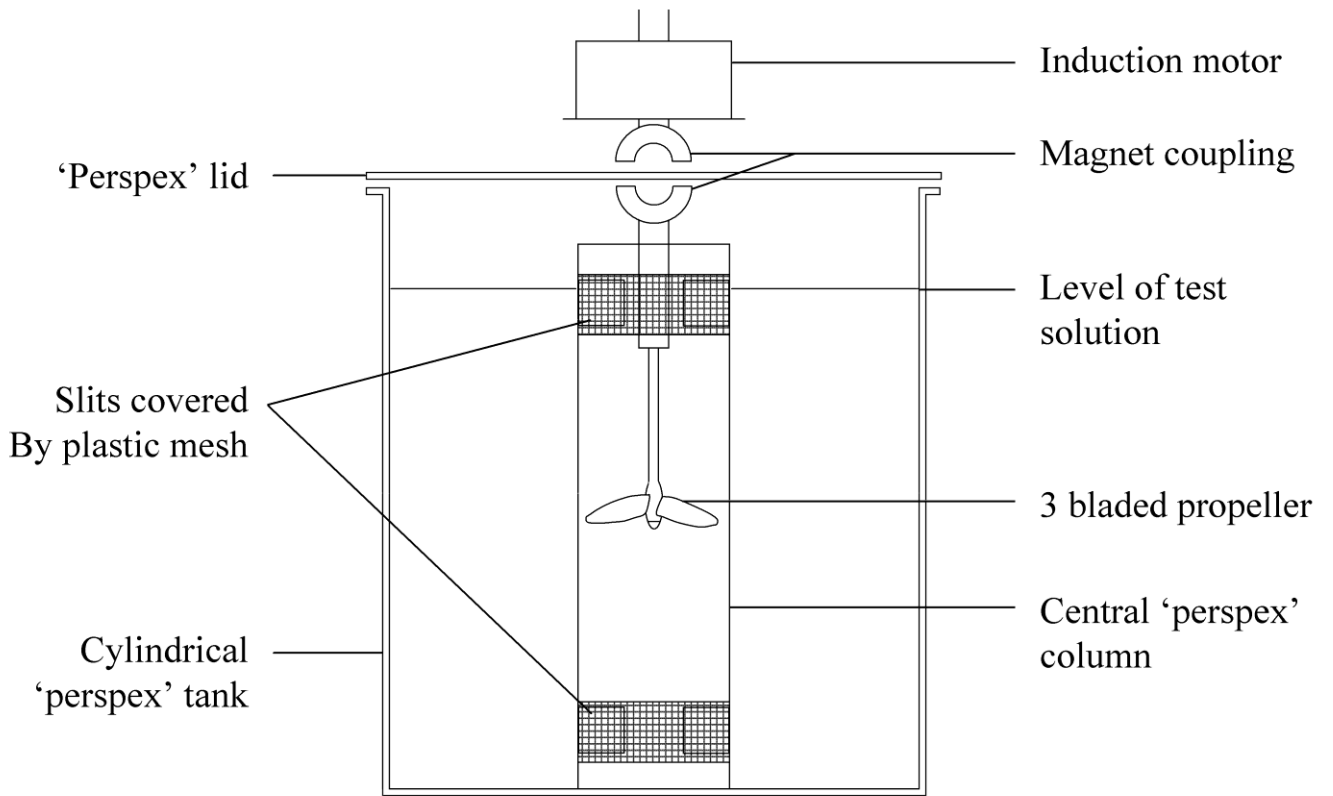
736 **Table 5:** Bioaccumulation factor (BAF = [total PAH] in fish muscle / [total PAH] in sea  
737 water) for CD1 (Chemically Dispersed oil using dispersant 1), CD2 (Chemically Dispersed  
738 oil using dispersant 2), MD (Mechanically Dispersed oil) and WSF (Water Soluble Fraction  
739 of oil) exposures. Respecting Quade test procedure, values obtained for each exposure  
740 conditions at several % of stock solution are considered as repeated measure. \*indicates  
741 significant differences (P<0.05) of BAF between exposure conditions. n.c. = not calculated.

% of stock solution	MD	CD1	CD2	WSF
0	n. c.	n. c.	n. c.	n. c.
2.4	33.1	75.1	61.4	16.1
12	42.5	98.4	66.7	16.5
18	58.2	99.3	141.4	20.6
24	126.9	98.1	59.2	12.1

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745 **Figure 1:** One tank of the experimental system (composed of twelve tanks) devised to

746 maintain a mixture of oil-dispersant droplets throughout the water column

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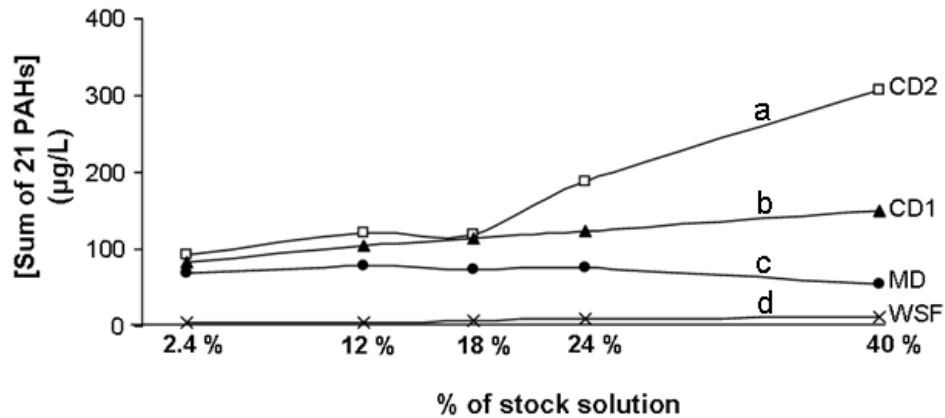
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755 **Figure 2:** Concentration of the sum of 21 PAHs (alkylated and parents) in sea water during  
 756 CD1 (Chemically Dispersed oil using dispersant 1), CD2 (Chemically Dispersed oil using  
 757 dispersant 2), MD (Mechanically Dispersed oil) and WSF (Water Soluble Fraction of oil).

758 The 21 PAHs represent the 16 US-EPA PAHs and five supplementary PAHs

759 (benzo[*b*]thiophene, biphenyl, dibenzothiophene, benzo[*e*]pyrene, perylene).

760 Values represent means of two measurements (at T = 0 h and at T = 24 h). To determine

761 whether the curves differed significantly, Quade test was conducted. Different letters above

762 curves indicates that curves differed significantly (P<0.05)

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