

**Toxicological effects of crude oil and oil-dispersant:  
biomarkers in the heart of the juvenile golden grey  
mullet (*Liza aurata*).**

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1 Toxicological effects of crude oil and oil-dispersant:  
2 biomarkers in the heart of the juvenile golden grey  
3 mullet (*Liza aurata*).

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27

28 **Abstract**

29

30 Dispersant use is a controversial oil spill response technique in coastal areas. Using an  
31 experimental approach, this study evaluated the toxicity of dispersant use upon juveniles of  
32 golden grey mullet (*Liza aurata*). Fish were exposed for 48 hours to either dispersant only,  
33 chemically dispersed oil, mechanically dispersed oil, the water-soluble fraction of oil or to  
34 control conditions. Following exposure and a depuration period, biomarkers were assessed in  
35 fish hearts, namely the total glutathione content and the activity of four enzymes (glutathione  
36 S-transferase, superoxide dismutase, catalase and glutathione peroxidases). Comparing  
37 biomarker responses between the different treatments, this study revealed that 48 hours  
38 exposure to dispersed oil (whether mechanically or chemically dispersed) resulted in a  
39 toxicity that was still detectable after a 14 days depuration period. Comparing biomarkers  
40 responses after an exposure to chemically and mechanically dispersed oil, this study suggests  
41 that chemical dispersion of the oil slick would not be more toxic than its natural dispersion  
42 under certain turbulent meteorological conditions (e.g. waves). Furthermore, the results  
43 indicated that the heart could be a target organ of interest in further studies investigating the  
44 toxicity of hydrocarbons. This study, which has been integrated into the DISCOBIOL project  
45 (Dispersant et techniques de lutte en milieu côtier: effets biologiques et apport à la  
46 réglementation), presents information of interest when attempting to provide a framework for  
47 dispersant applications in coastal areas.

48

49 **Key words:** petroleum, dispersant, antioxidant enzyme, glutathione, heart, *Liza aurata*

50

51

52 **1. Introduction**

53

54 Oil spill response techniques have been developed to remediate the ecological consequences  
55 of petroleum released into the environment. Dispersant spreading, by boat and/or by plane, is  
56 a commonly employed method. According to Chapman *et al.* (2007), chemical dispersants  
57 were used on 18% of oil spills between 1995 and 2005. This was mainly due to the  
58 environmental benefits provided by dispersant application (such as shown in Baca *et al.*,  
59 2005). Indeed, by inducing the transformation of an oil slick on the sea surface into oil  
60 droplets in the water column, dispersants: (i) enhance the bacterial degradation of petroleum  
61 (Thiem, 1994; Churchill *et al.*, 1995; Swannell and Daniel, 1999); (ii) rapidly dilute the  
62 petroleum in the water column, preventing drifting of the oil slick into ecologically relevant  
63 shoreline habitats (Merlin, 2005); and (iii) reduce the risk of contamination of organisms  
64 dwelling on the sea surface (e.g. seabirds and marine mammals).

65 In spite of these advantages, certain restrictions are placed on the application of dispersants in  
66 European coastal areas. These precautions are based on the low dilution potential of shallow  
67 waters in such areas, where the dispersant use could induce high concentrations of  
68 hydrocarbons in the water column and, consequently, toxic conditions for aquatic organisms  
69 in those areas. This increase in toxicity has been highlighted in many experimental studies.  
70 For instance, biomarker approaches demonstrated toxicity in *Platichthys flesus* (Baklien *et al.*,  
71 1986), *Salmo salar* (Gagnon and Holdway, 2000), *Macquaria novemaculeata* (Cohen *et al.*,  
72 2001) and *Sebastes schlegeli* (Jung *et al.*, 2009) following dispersant use. However, these  
73 experimental studies considered the toxicity of chemically enhanced water-accommodated  
74 fractions (CEWAF; described in Singer *et al.*, 2000), which did not contain the vast quantities  
75 of oil droplets formed *in situ* during the dispersion of an oil slick. Moreover, recent  
76 advancements in chemistry have enabled the creation of new dispersant formulations. These

77 so-called ‘third generation’ dispersants are, in contrast to previous generations, considered  
78 intrinsically non-toxic and biodegradable (Merlin, 2005).

79 In this new context, a study investigating the toxicity of dispersed oil, considering both the  
80 recent ‘third generation’ dispersant formulations and also the presence of oil droplets in the  
81 water column, would be of interest. As a consequence, the DISCOBIOL project (Dispersant et  
82 techniques de lutte en milieu côtier: effets biologiques et apport à la réglementation) was  
83 created to provide information concerning the environmental impact of dispersed oil in  
84 coastal areas. The aim of this project was to investigate the potential toxicity and advantages  
85 of dispersant use in coastal waters in order to develop a framework for policies on dispersant  
86 use. This study evaluates the toxicity of dispersant use in coastal areas by means of an  
87 experimental approach. Using a turbulent experimental system, the toxicity of dispersant  
88 application was evaluated, taking into account the presence in coastal areas of mixing  
89 processes (e.g. waves) which are required for dispersant use (Merlin, 2005). Moreover, to  
90 simulate current oil dispersant application, our study used a third generation dispersant, which  
91 is the most recent and commonly used of formulations (Merlin, 2005) and is considered as  
92 less toxic and more concentrated in tensio-active components than earlier ones. The oil used  
93 was a Brut Arabian crude oil considering the important importation of Arabian oils in the  
94 European Union in 2011 (European commission directorate-general for energy,  
95 [http://ec.europa.eu/energy/observatory/oil/import\\_export\\_en.htm](http://ec.europa.eu/energy/observatory/oil/import_export_en.htm)) and thus its transport along  
96 the European coast.

97 The present study is a part of the DISCOBIOL project. Employing a biomarker approach, it  
98 investigated the toxicity of dispersed oil upon juveniles of a pelagic fish species, the golden  
99 grey mullet (*Liza aurata*). This species was chosen because of its significant role in coastal  
100 ecosystems, facilitating the transportation of important particulate organic matter from salt  
101 marshes to marine coastal waters (Laffaille *et al.*, 1998). Gautier and Hussenot (2005) showed

102 the presence of this species in coastal areas during the early part of its life, thus juvenile  
103 organisms were chosen.

104 This ecotoxicological study was conducted at the organ level, on the hearts of fish, since: (i)  
105 the toxicological effects of contaminants upon this organ are likely to alter fish survival, a  
106 consequence of the heart being a central organ involved in many physiological,  
107 immunological and metabolic functions, moving substances such as gases, nutrients, wastes,  
108 hormones, antibodies and heat; and (ii) several studies have previously investigated the  
109 toxicological effect of hydrocarbons upon this organ, which could, therefore, be considered as  
110 a target organ of interest. For instance, Incardona et al. (2004, 2009) showed cardiac function  
111 defects in fish embryos exposed to hydrocarbons and recently Hicken et al. (2011) showed  
112 that sublethal exposure to crude oil during embryonic development alters cardiac morphology.  
113 In order to illustrate the toxicological effects of dispersed oil upon the heart, a biomarker  
114 approach was used, focussing on the detoxification processes, but also on responses to  
115 oxidative stress induced by petroleum hydrocarbons. After exposure to contaminants,  
116 oxidative stress is provoked by the formation of reactive oxygen species (ROS; Van der Oost  
117 *et al.*, 2003). This phenomenon is likely to induce a loss of structural and/or functional  
118 integrity in proteins, lipids and DNA (Winston and Di Giulio, 1991). Consequently, the  
119 biomarkers examined during this study were: total glutathione, which is involved in the  
120 contaminant detoxification processes (Maracine and Segner, 1998); glutathione S-transferase,  
121 which is involved in the detoxification processes and also in antioxidant responses (Van der  
122 Oost *et al.*, 2003); and superoxide dismutase, catalase and glutathione peroxidase, which  
123 indicate antioxidant responses in hydrocarbon-contaminated fish (Pan *et al.*, 2005; Oliveira *et*  
124 *al.*, 2008; Nahrgang *et al.*, 2009; Hannam *et al.*, 2010). These biomarkers were considered  
125 suitable for this investigation as many previous studies have shown them to respond to  
126 petroleum contamination and especially to the PAHs (polycyclic aromatic hydrocarbons)

127 contained in petroleum (Avci *et al.*, 2005; Almroth *et al.*, 2008; Oliveira *et al.*, 2008; Jung *et*  
128 *al.*, 2009; Kopecka-Pilarczyk and Correia, 2009; Nahrgang *et al.*, 2009)

129

## 130 **2. Materials and methods**

131

### 132 2.1. Chemicals

133

134 A Brut Arabian crude oil, containing 54% saturated hydrocarbons, 34% aromatic  
135 hydrocarbons and 12% polar compounds, was used for this study. This composition was  
136 evaluated by CEDRE (Centre of Documentation, Research and Experimentation on accidental  
137 water pollution, Brest, France), a laboratory certified according to ISO 9001 and ISO 14001.

138 The API (American Petroleum Institute) gravity of the oil was 33.

139

140 The dispersant, manufactured by Total Fluides and designed for marine environment, was  
141 evaluated by CEDRE and was deemed effective enough to be used during oil spills  
142 (preliminarily determined using the NF.T.90-345 method, see Bocard, 1986 for a more  
143 detailed description of this method), non-toxic at the concentration recommended by the  
144 manufacturer (preliminarily determined using the standard toxicity test NF.T.90-349) and  
145 biodegradable. This dispersant is composed of surfactants (surface active agents) and  
146 solvents. As it is a 'third generation' dispersant, the surfactants are blends of anionic and non-  
147 ionic types. The manufacturer states that the chemical compounds representing a human and  
148 aquatic ecosystems health risk are non-ionic surfactants (24%) and anionic surfactants  
149 (between 12 and 24%).

150

### 151 2.2. Experimental animals

152

153 One hundred *Liza aurata* juveniles were used for the experiment. They were provided by  
154 Commercio Pesca Novellame Srl (Chioggia, Italy). The fish were acclimatized for 3 weeks in  
155 300 L through-flow tanks containing seawater provided by Oceanopolis (Brest, France)  
156 (dissolved oxygen:  $91 \pm 2\%$ ; salinity:  $35 \pm 1\%$ ; temperature:  $15 \pm 0.1^\circ\text{C}$ ; pH:  $8.01 \pm 0.04$ ; a  
157 photoperiod of 12 hours light/12 hours dark; in seawater free of nitrates and nitrite). During  
158 acclimatization they were fed daily with fish food (Neosupra AL3 from Le Gouessant  
159 Aquaculture), but were starved for 48 hours prior to the bioassays, and throughout the  
160 exposure period, in order to avoid bile evacuation from the gallbladder. At the end of the  
161 acclimatization period, just before exposure, their average length was  $140.3 \pm 0.11$  mm (mean  
162  $\pm$  standard error of the mean, s.e.m.) and their average weight was  $38.18 \pm 1.28$  g.

163

### 164 2.3. Experimental system

165

166 The experimental system used, as previously described by Milinkovitch *et al.* (2011a), was in  
167 a temperature controlled room ( $15^\circ\text{C}$ ). Thus the water temperature for the experiment was  $15$   
168  $\pm 0.1^\circ\text{C}$ . Other physicochemical parameters were also measured: pH ( $8.02 \pm 0.07$ ) and  
169 dissolved oxygen ( $95 \pm 1\%$ ) levels remained constant throughout the study. Water was not  
170 renewed throughout the experiment. The system was devised to maintain the oil and  
171 dispersant mixture as a homogenous solution. Circulation and homogenization of solution in  
172 the 300 L seawater tank was ensured by a funnel linked to a Johnson L450 pump. The  
173 solution is sucked up in the funnel, creating a vortex at the surface of the tank, and then  
174 expelled at the bottom of the tank after passed through the pump.

175

### 176 2.4. Exposure treatments



177

178 Five exposure treatments were prepared, respecting a protocol previously described in  
179 Milinkovitch et al. (2011a, b, 2012). The control exposure treatment (C) was made using  
180 seawater provided by Oceanopolis (Brest, France). The mechanically dispersed (MD) oil  
181 exposure treatment was made by pouring 20 g of petroleum in the funnel of the experimental  
182 system. The chemically dispersed (CD) oil exposure treatment was made by pouring 20 g of  
183 petroleum in the funnel, as for MD exposure, and 1 g of dispersant (respecting the 5 % of  
184 dispersant recommended by the manufacturer). The dispersant (D) exposure treatment,  
185 representing an internal control for CD, was made by pouring 1 g of dispersant. For the water-  
186 soluble fraction of the oil (WSF), in addition to the funnel and the pump, which were kept to  
187 maintain the same level of water agitation and circulation as for the other treatments, a 20 g  
188 oil slick was contained using a plastic cylinder (21 cm diameter). This treatment differed from  
189 the standardized water accommodated fraction (WAF) treatment described by Singer et al.  
190 (2000). It was designed to simulate the containment of the oil slick before recovery which is  
191 an alternative response technique to dispersant use. During the entire exposure period, the oil  
192 slick remained at the surface without mixing and consequently no droplets were observed in  
193 the water column, suggesting that the fish were only exposed to the soluble fraction of the oil.  
194 For all treatments, the weight of the chemicals masses delivered to the experimental system  
195 were determined precisely by mass difference. Twenty grams of oil and 1 g of dispersant were  
196 taken from beakers and delivered (using a glass syringe, VWR international) into the vortex  
197 of water created by the funnel linked to the pump.

198 Although the solutions remained homogenous (less than 5% difference between three Total  
199 petroleum hydrocarbons concentration measurements sampled at three different depths within  
200 the experimental tanks), no fish were exposed within 24 hours of the solutions being made.  
201 Thereafter, groups of 10 fish were randomly placed in the five experimental tanks ( $T_0$ ), which

202 each contained one of the exposure treatments described above. The fish were exposed to the  
203 various treatments for a period of 48 hours and no fish mortality was observed. The protocol  
204 (from the preparation of the exposure treatment to the fish exposure) was replicated, so that a  
205 total of 20 fish were subjected to each exposure treatment.

206 At the end of the exposure period (**A**), five fish per tank were euthanised using eugenol (4-  
207 allyl-2-methoxyphenol), while the other five fish were placed in uncontaminated water and  
208 fed for 14 days (**B**). This was performed for both sets of replicates, so that 10 fish per  
209 exposure treatment (C, CD, MD, WSF or D) were euthanised immediately after exposure and  
210 10 fish per treatment were euthanised following the depuration period.

211 From fish exposed, the hearts were removed and stored at -80°C prior to analysis. The liver  
212 and blood were removed and analysed as part of another study (Milinkovitch *et al.*, 2011a) in  
213 which information concerning Total petroleum hydrocarbons (TPHs) and PAH concentrations  
214 in seawater is available. Thus, the information concerning the present study water  
215 contamination is available and discussed in Milinkovitch *et al.* (2011a) and also summarized  
216 in Table 1.

217

## 218 2.5. Measurement of biomarker responses in fish hearts

219

220 The hearts were ground in a phosphate buffer (100 mM, pH 7.8) containing 20% glycerol and  
221 0.2 mM phenylmethylsulfonyl fluoride as a serine protease inhibitor. The homogenates were  
222 then centrifuged at 10,000 *g* and 4°C for 15 minutes to obtain the post-mitochondrial fraction  
223 (PMF). Total protein concentrations were determined using the method of Bradford (1992),  
224 with bovine serum albumin (Sigma-Aldrich Chemicals, France) as the standard. Prior to the  
225 biomarker response measurements, all assays were optimised for juvenile *Liza aurata* heart  
226 samples and adapted for use on a microplate.

227 Total glutathione (GSHt) concentrations were measured according to the method of  
228 Vandeputte *et al.* (1994). In brief, 10  $\mu$ l of each TCA-deproteinised sample was mixed with a  
229 phosphate buffer (100 mM, pH 7.5) containing 10 mM EDTA, 0.3 mM NADPH and 1 mM  
230 Ellman reagent. The enzymatic reaction was spectrophotometrically monitored at 405 nm and  
231 the results were expressed in  $\mu$ mol of GSHt/g of protein.

232 Glutathione S-transferase (GST) activity assays were conducted according to the method of  
233 Habig *et al.* (1974). In brief, 20  $\mu$ g of PMF proteins were mixed with a phosphate buffer (100  
234 mM, pH 6.5) containing 1 mM chloro dinitrobenzene and 1 mM reduced glutathione. The  
235 enzymatic reaction was spectrophotometrically monitored at 340 nm and the results were  
236 expressed in U of GST/g of protein.

237 Superoxide dismutase (SOD) activity was measured using the assay developed by Paoletti *et*  
238 *al.* (1986). In brief, the inhibition of NADH (350  $\mu$ M) oxidation by 11  $\mu$ g of PMF proteins  
239 was monitored at 340 nm. For this purpose, PMF proteins were added to a phosphate buffer  
240 (100 mM, pH 7.5) with 0.4 mM NADH, 6 mM EDTA and 3 mM  $\text{MnCl}_2$ . The results were  
241 expressed in U of SOD/mg of protein.

242 Catalase (CAT) activity was monitored using the method previously described by Babo and  
243 Vasseur (1992). In brief, 5  $\mu$ g of PMF proteins were mixed (v:v) with a phosphate buffer (100  
244 mM, pH 7.5) and 28 mM hydrogen peroxide. The hydrogen peroxide degradation kinetics  
245 were assessed at 280 nm and the results were expressed in U of CAT/mg of protein.

246 Glutathione peroxidase (GPx) activity was determined using 30  $\mu$ g of PMF proteins according  
247 to the method of Paglia and Valentine (1967). The PMF proteins were added to a phosphate  
248 buffer (100 mM, pH 7.6) with 2 mM reduced glutathione, 0.12 mM NADPH and 2 U/ml  
249 glutathione reductase. Cumene hydroperoxide (0.55 mM) was used as the substrate and  
250 enzymatic activity was assessed at 340 nm. The results were expressed in U of GPx/g of  
251 protein.

252

## 253 2.6. Statistical analysis

254

255 Statistical analyses were carried out using Statistica software (version 9). For each variable  
256 (GSHt, GST, SOD, CAT and GPx activities) and each treatment (C, CD, MD, WSF and D)  
257 separately, no significant difference was found (determined with t-tests) between replicates of  
258 experiment (described in 2.4.). Normality and homoscedasticity were verified using the  
259 Kolmogorov-Smirnov and Cochran tests. For the GSHt concentrations and the GST, SOD,  
260 CAT and GPx activities, two-way factorial analyses of variance (ANOVA) were performed,  
261 in order to assess the effects of the different exposure treatments (C, CD, MD, WSF and D)  
262 after exposure and depuration. For each biological parameter, ANOVA was followed by  
263 planned comparison tests (Tukey's HSD test) to detect any significant differences between  
264 contamination after exposure and after the depuration period. The significance of the results  
265 was ascertained at  $\alpha=0.05$ . The results were expressed as means  $\pm$  s.e.m. (standard error of the  
266 mean) of 10 individual fish (n=10).

267

## 268 3. Results

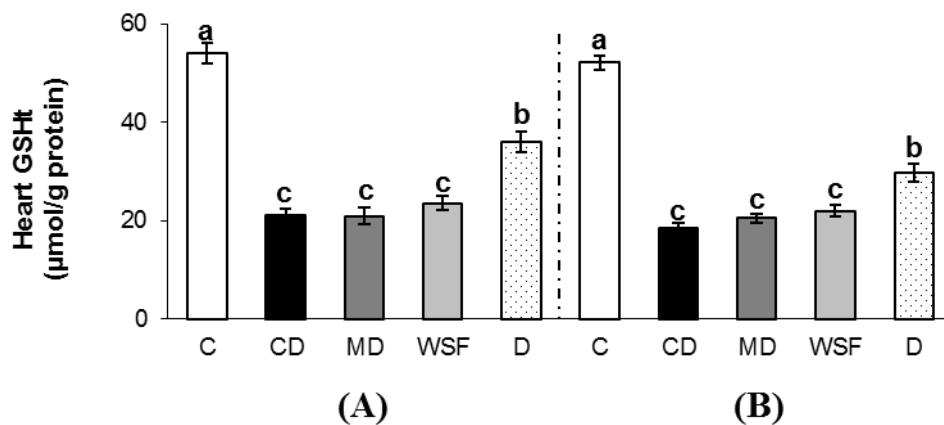
269

### 270 3.1. Total glutathione (GSHt) concentrations in fish hearts

271

272 The means of the GSHt concentrations, based on 10 individual analyses, showed values of  
273  $54.0 \pm 0.2$   $\mu\text{mol}$  of GSHt/g of protein (Figure 1) for control *Liza aurata*, (C) at the end of the  
274 48 hour exposure period (A). The GSHt concentrations in fish hearts showed a significant  
275 decrease when the fish were exposed to any of the contaminants (WSF, MD, CD or D). GSHt  
276 levels after WSF, MD or CD exposures were half that of the control. The GSHt level

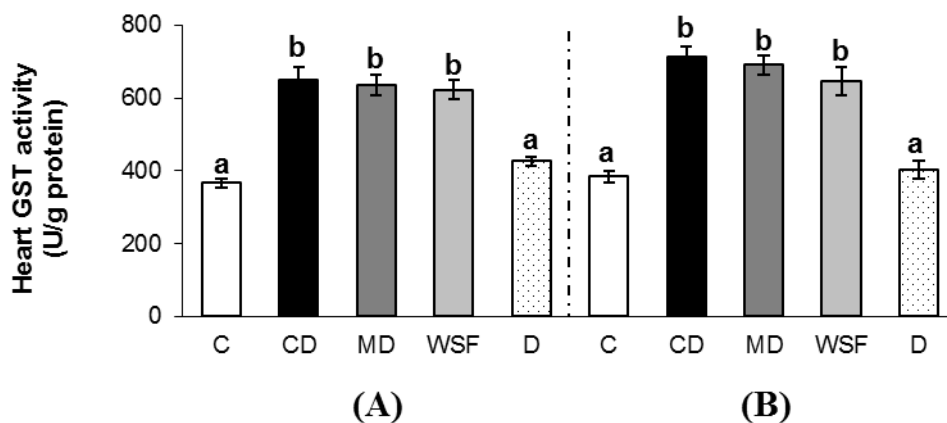
277 following dispersant (D) exposure was significantly lower than that of the control, but  
 278 significantly higher than GSht levels assessed after WSF, MD or CD exposures.  
 279 Following the 14 day depuration period (B), GSht levels were not significantly different from  
 280 those observed immediately after exposure (A). Concentration patterns also remained  
 281 unchanged: the hearts of fish exposed to either WSF, MD or CD showed lower concentrations  
 282 of glutathione and the hearts of fish exposed to the dispersant (D) showed significantly lower  
 283 levels of glutathione when compared to those exposed to the control treatment, but higher  
 284 levels than those exposed to WSF, MD or CD.



285  
 286 **Figure 1:** Concentration of total glutathione (GSht) in the heart of *Liza aurata*. Glutathione  
 287 concentration was measured after 48 h exposure (A) to Control (C), Chemically Dispersed oil (CD),  
 288 Mechanically Dispersed oil (MD), Water Soluble Fraction (WSF) and Dispersant (D) exposure  
 289 treatments; and after 14 days of depuration (B) following the exposure: Control (C), Chemically  
 290 Dispersed oil (CD), Mechanically Dispersed oil (MD), Water Soluble Fraction (WSF) and Dispersant  
 291 (D). Values represent mean  $\pm$  standard error of ten individual analyses (n=10 per treatment). For  
 292 each period (after 48h exposure and after 14 days of depuration), different letters above bars (a, b, c)  
 293 indicate a significant difference (where  $P < 0.05$ ). a indicates values significantly not different from  
 294 control (C) values. b indicates values significantly different from control (C) values. c indicates values  
 295 significantly different from a and b. For each exposure treatment, \* indicates a significant difference  
 296 ( $P < 0.05$ ) between the values obtained after 48 h exposure and the values obtained after 14 days of  
 297 depuration.

298  
 299 3.2. Glutathione S-transferase (GST) activity in fish hearts

301 GST activity (Figure 2) in the hearts of the control fish (C) was  $366.9 \pm 12.1$  U/g of protein at  
 302 the end of the 48 hour exposure period (A). No significant increase was observed in fish  
 303 exposed to the dispersant (D). A significant increase in the activity of this enzyme was  
 304 observed in fish that were exposed to hydrocarbons: a 70, 72, and 78 % increase for WSF,  
 305 MD and CD, respectively. Indeed, compared to the control treatment (C), heart GST activity  
 306 increased after the fish were exposed to either WSF, MD or CD. Following the 14 day  
 307 depuration period (B), GST activity levels were still higher in those fish that had been  
 308 exposed to WSF, MD or CD.  
 309

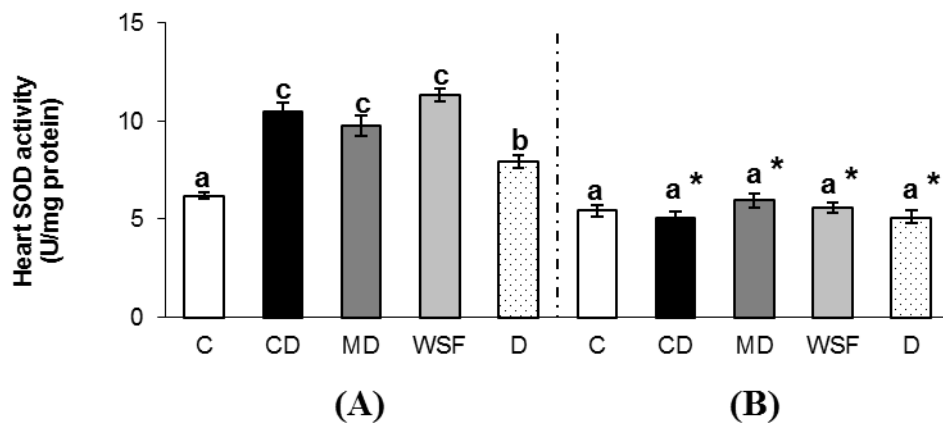


310  
 311 **Figure 2:** Glutathione-S-Transferase (GST) activity in the heart of *Liza aurata*. Activity was measured  
 312 after 48 h exposure (A) to Control (C), Chemically Dispersed oil (CD), Mechanically Dispersed oil  
 313 (MD), Water Soluble Fraction (WSF) and Dispersant (D) exposure treatments; and after 14 days of  
 314 depuration (B) following the exposure: Control (C), Chemically Dispersed oil (CD), Mechanically  
 315 Dispersed oil (MD), Water Soluble Fraction (WSF) and Dispersant (D). Values represent mean  $\pm$   
 316 standard error of ten individual analyses (n=10 per treatment). For each period (after 48h exposure  
 317 and after 14 days of depuration), different letters above bars (a, b, c) indicate a significant difference  
 318 (where  $P < 0.05$ ). a indicates values significantly not different from control (C) values. b indicates  
 319 values significantly different from control (C) values. c indicates values significantly different from a  
 320 and b. For each exposure treatment, \* indicates a significant difference ( $P < 0.05$ ) between the values  
 321 obtained after 48 h exposure and the values obtained after 14 days of depuration  
 322

### 323 3.3. Superoxide dismutase (SOD) activity in fish hearts

324

325 SOD activity in the hearts of the control (C) *Liza aurata* was  $6.2 \pm 0.2$  U/mg of protein at the  
 326 end of the 48 hour exposure period (A). SOD activity exhibited a significant increase when  
 327 fish were exposed to any of the contaminants (WSF, MD, CD or D). For WSF, MD and CD, a  
 328 significant 82, 52, 70% increase in SOD activity, was observed (Figure 3), respectively. For  
 329 the dispersant (D), a 28% increase in SOD activity was observed, compared to control values.  
 330 This activity was significantly lower than that measured after WSF, MD or CD exposure.  
 331 Following the 14 day depuration period (B), a significant decrease in the activity of this  
 332 enzyme was observed and activity levels of SOD returned to the control values for all  
 333 exposure treatments.



334  
 335 **Figure 3:** SuperOxide Dismutase (SOD) activity in the heart of *Liza aurata*. Activity was measured  
 336 after 48 h exposure (A) to Control (C), Chemically Dispersed oil (CD), Mechanically Dispersed oil  
 337 (MD), Water Soluble Fraction (WSF) and Dispersant (D) exposure treatments; and after 14 days of  
 338 depuration (B) following the exposure: Control (C), Chemically Dispersed oil (CD), Mechanically  
 339 Dispersed oil (MD), Water Soluble Fraction (WSF) and Dispersant (D). Values represent mean  $\pm$   
 340 standard error of ten individual analyses (n=10 per treatment). For each period (after 48h exposure  
 341 and after 14 days of depuration), different letters above bars (a, b, c) indicate a significant difference  
 342 (where  $P < 0.05$ ). a indicates values significantly not different from control (C) values. b indicates  
 343 values significantly different from control (C) values. c indicates values significantly different from a  
 344 and b. For each exposure treatment, \* indicates a significant difference ( $P < 0.05$ ) between the values  
 345 obtained after 48 h exposure and the values obtained after 14 days of depuration.

346  
 347

348

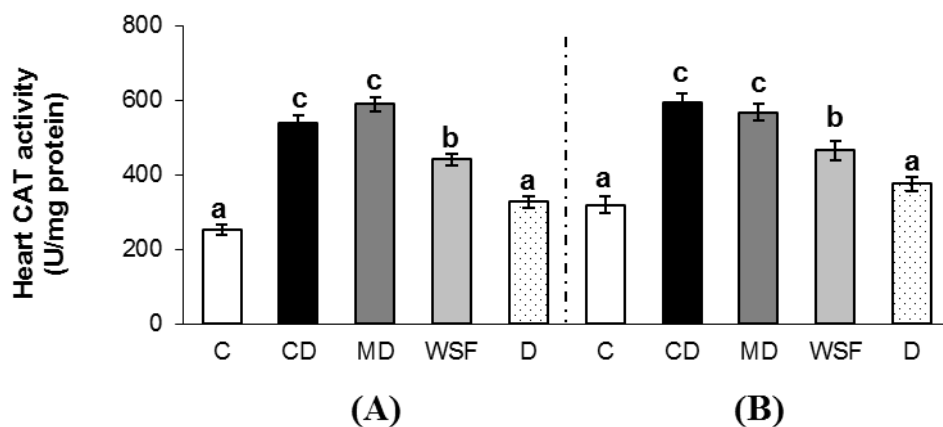
349

350 3.4. Catalase (CAT) activity in fish hearts

351

352 CAT activity (Figure 4) in the hearts of the control (C) *Liza aurata* was  $253.3 \pm 13.3$  U/mg of  
353 protein at the end of the 48 hour exposure period (A). Our results showed that the activity of  
354 this enzyme was significantly increased by WSF, MD and CD exposures. The increase was  
355 significantly higher ( $p < 0.05$ ) for fish exposed to CD or MD (112% and 132% increase  
356 compared to control values, respectively) than for fish exposed to WSF or D (74% and 29%  
357 increase compared to control values, respectively). Furthermore, the increase in activity was  
358 significantly higher for WSF than for D.

359 After the 14 day depuration period (B), CAT activity remained unchanged for all exposure  
360 treatments (when compared to the levels observed immediately after exposure). The patterns  
361 of CAT activity also remained unchanged: CD and MD exposure induced significantly higher  
362 CAT activity, and CAT activity was significantly greater in fish exposed to WSF than in fish  
363 exposed to D.



364

365 **Figure 4:** Catalase (CAT) activity in the heart of *Liza aurata*. Activity was measured after 48 h  
366 exposure (A) to Control (C), Chemically Dispersed oil (CD), Mechanically Dispersed oil (MD), Water  
367 Soluble Fraction (WSF) and Dispersant (D) exposure treatments; and after 14 days of depuration (B)



368 following the exposure: Control (C), Chemically Dispersed oil (CD), Mechanically Dispersed oil (MD),  
 369 Water Soluble Fraction (WSF) and Dispersant (D). Values represent mean  $\pm$  standard error of ten  
 370 individual analyses (n=10 per treatment). For each period (after 48h exposure and after 14 days of  
 371 depuration), different letters above bars (**a**, **b**, **c**) indicate a significant difference (where  $P < 0.05$ ). **a**  
 372 indicates values significantly not different from control (C) values. **b** indicates values significantly  
 373 different from control (C) values. **c** indicates values significantly different from **a** and **b**. For each  
 374 exposure treatment, \* indicates a significant difference ( $P < 0.05$ ) between the values obtained after  
 375 48 h exposure and the values obtained after 14 days of depuration.

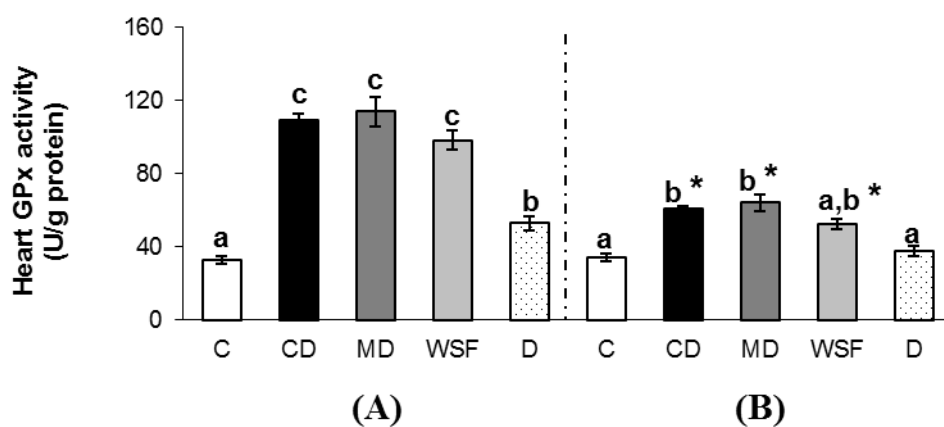
376

### 377 3.5. Glutathione peroxidase (GPx) activity in fish hearts

378

379 At the end of the exposure period (**A**), the GPx activity (Figure 5) was  $32.8 \pm 2.2$  U/g of  
 380 protein in the hearts of the control fish (C). GPx activity displayed the similar pattern of  
 381 results as those observed for SOD activity: a significant increase in all exposure groups,  
 382 although this increase was significantly less intense for the D exposure treatment (65%) than  
 383 for the other treatments (WSF 206%, CD 240% and MD 253%). After depuration (**B**), a  
 384 significant decrease in activity was observed in the hearts of fish exposed to WSF, CD or MD.  
 385 However, in spite of this decrease, GPx activity was still higher in fish exposed to CD and  
 386 MD than in the control.

387



388

389 **Figure 5:** Glutathion peroxidase (GPx) activity in the heart of *Liza aurata*. Activity was measured after  
390 48 h exposure **(A)** to Control (C), Chemically Dispersed oil (CD), Mechanically Dispersed oil (MD),  
391 Water Soluble Fraction (WSF) and Dispersant (D) exposure treatments; and after 14 days of  
392 depuration **(B)** following the exposure: Control (C), Chemically Dispersed oil (CD), Mechanically  
393 Dispersed oil (MD), Water Soluble Fraction (WSF) and Dispersant (D). Values represent mean  $\pm$   
394 standard error of ten individual analyses (n=10 per treatment). For each period (after 48h exposure  
395 and after 14 days of depuration), different letters above bars (**a**, **b**, **c**) indicate a significant difference  
396 (where  $P < 0.05$ ). **a** indicates values significantly not different from control (C) values. **b** indicates  
397 values significantly different from control (C) values. **c** indicates values significantly different from **a**  
398 and **b**. For each exposure treatment, \* indicates a significant difference ( $P < 0.05$ ) between the values  
399 obtained after 48 h exposure and the values obtained after 14 days of depuration.

400

## 401 **4. Discussion**

402

### 403 4.1. Total glutathione content and GST activity in fish hearts

404

405 The results of this study indicated a decrease in GSht concentrations in fish hearts following  
406 all exposure treatments. This decrease of glutathione content has also been observed in the  
407 livers of teleost fish after exposure to PAHs (Almroth *et al.*, 2008). However, to our  
408 knowledge, few studies have investigated the modulation of total glutathione content in the  
409 hearts of fish, making comparison between these results and the literature difficult.

410 Since total glutathione is considered to be an important molecule in cellular defence against  
411 contaminants (Maracine and Segner, 1998), the results of this study suggest that the potential  
412 for fish to cope with contaminants is impaired after exposure to WSF, CD, MD or D.  
413 Moreover, the results obtained following the depuration period indicated that, in the case of  
414 all contaminations, GSht concentrations had not increased to reach control levels, suggesting  
415 repletion had still not occurred after 14 days of depuration. Consequently, even after the  
416 depuration period, the potential for the fish to cope with further contaminated environments,  
417 such as those present in coastal areas, was still impaired.

418 Comparing GSht levels for the different exposure treatments, our results showed a more  
419 profound depletion for CD, MD and WSF exposures than for D, suggesting that the  
420 toxicological effects of the three exposure treatments containing hydrocarbons were more  
421 intense. In parallel, no significant differences between CD, MD and WSF exposures were  
422 indicated, suggesting, on the basis of this single result, that the dispersion of the oil, whether  
423 mechanically or chemically, did not increase the toxicological effects of the petroleum on the  
424 concentration of GSht.

425

426 During contamination, several detoxification processes could involve the use of glutathione.  
427 In our study, three hypotheses could explain the decrease in total glutathione: (i) it could be  
428 due to an inhibition of the GSH synthesis rate by contaminants, as suggested in Zhang *et al.*  
429 (2004) in goldfish (*Carassius auratus*); (ii) the heavy metals in petroleum (mainly vanadium  
430 and nickel; Salar Amoli *et al.*, 2006) could be chelated by reduced glutathione, leading to GS-  
431 V or GS-Ni-binding complexes (Sies, 1999), which cannot be assessed through biochemical  
432 analysis and consequently contribute to the observed decrease in total glutathione content;  
433 (iii) after a phase 1 biotransformation induced by EROD (ethoxyresorufin-O-deethylase)  
434 activity, the GST could induce a conjugation of the oxidized PAHs to reduced glutathione  
435 (George, 1994). The conjugate could then be excreted, thus leading to a decrease in total  
436 glutathione. For dispersant alone (D) the decrease of total glutathione can only be explained  
437 by the first hypothesis since dispersant alone contains neither PAHs nor heavy metals. Thus,  
438 the reduction of glutathione could be due to the inhibition of the GSH synthesis rate by the  
439 dispersant. For fish exposed to CD, MD and WSF, all hypotheses are conceivable. However,  
440 our results suggest an intense role of the PAH conjugation. Indeed, for these exposure  
441 treatments, the decrease in total glutathione was associated with an increase in GST activity.

442 Moreover, the regression between GST activity and glutathione content was tested (data not  
443 shown) and found highly significant ( $P < 0.001$ ).

444 Comparing the biological effects with the seawater PAH concentrations, this study showed  
445 same magnitude of response in the GST activity levels between WSF and either CD and MD  
446 treatments while total concentrations of PAHs in CD and MD treatments are both  
447 approximately 8 and 50 times higher than in WSF, at the beginning ( $T_0 = 0$  h) and at the end of  
448 the exposure ( $T_1 = 48$  h) respectively. This could be due to the fact that GST activity in heart  
449 reached its maximum with WSF treatment and that consequently a higher load of  
450 contamination (through CD and MD exposure) did not induce a further increase of GST  
451 activity. Since GST activity is involved in the depletion of total glutathione, the same  
452 magnitude of response of this enzyme for WSF, CD and MD could explain the identical GSht  
453 depletion observed for these three treatments.

454

#### 455 4.2. Activity of antioxidant enzymes in fish hearts

456

457 The results of this study showed an increase in the three enzymes assessed after oil exposure,  
458 when compared to the control. This response was previously observed in several  
459 ecotoxicological studies and, more specifically, in studies that investigated the toxicological  
460 effects of hydrocarbons in teleost fish (Zhang *et al.*, 2004; Sun *et al.*, 2006; Oliveira *et al.*,  
461 2008; Oliva *et al.*, 2010). The enhancement of antioxidant enzyme activities is considered to  
462 be a defence mechanism against the production of reactive oxygen species (ROS), initiated by  
463 the incorporation of PAHs in the internal environment of organisms (Guengerich, 1993; Van  
464 der Oost *et al.*, 2003).

465 The antioxidant enzyme activities in fish hearts revealed that CD and MD exposures induced  
466 the highest antioxidant responses for SOD, CAT and GPx. These results are in accordance

467 with those of Milinkovitch *et al.* (2011a), since the PAH biliary metabolite concentrations  
468 (after 48 hours exposure) indicated that CD and MD also induced the highest levels of PAH  
469 incorporation. However, although biliary metabolites did not reveal a significant increase in  
470 PAH incorporation after WSF exposure in our previous investigation (Milinkovitch *et al.*,  
471 2011 a), the present study showed that the antioxidant response of SOD, GPx and CAT was  
472 significantly increased after WSF exposure. This finding suggests that antioxidant activity in  
473 fish heart could be a more sensitive biomarker than PAH biliary metabolites in the aim to  
474 detect the precocious biological effects of oil.

475 As previously described (discussing GST activity in 4.1.), the biological effects did not match  
476 with the seawater PAH concentrations measurements. Indeed, regarding to SOD and GPx  
477 activities, this study did not highlight significant different responses in the antioxidant  
478 activities between WSF and either CD and MD treatments while total concentrations of PAHs  
479 in CD and MD treatments are both 8 and 50 times higher than in WSF (at the beginning *i.e.*  
480 T0=0 h and at the end of the exposure *i.e.* T1=48 h, respectively). On this basis and  
481 analogously to GST activity, it is conceivable to hypothesize that antioxidant activities of  
482 SOD and GPx in heart reached their maximum with WSF treatment and that consequently a  
483 higher load of contamination (through CD and MD exposure) did not induce a further  
484 increase of antioxidant activity.

485 Regarding to CAT activity, results exposed the effects of oil dispersion (whatever  
486 mechanically or chemically) since, after depuration, CAT activity was higher for MD and CD  
487 exposures than for WSF exposure. However, this statement must be interpreted cautiously  
488 since it is based on a single biomarker analysis; 4 of the other 5 biomarkers (GSHt, GST,  
489 SOD, GPx) did not highlight significant difference between WSF and either CD and MD  
490 exposure.

491 After 14 days of depuration, (i) CAT activity was still significant following CD, MD or WSF  
492 exposure and (ii) GPx activity was still significant following CD and MD exposures. Thus, in  
493 addition to the transitory effects of hydrocarbons observed after acute exposure (48 hours),  
494 these results also illustrated the effect of oil after depuration.

495 Recent studies have suggested a relationship between the impairment of fish survival and the  
496 modulation of the antioxidant response. Allen and Moore (2004) revealed links between  
497 antioxidant response and the health status of organisms. Tsangaris *et al.* (2007) found a  
498 significant correlation between glutathione peroxidase and the scope for growth in *Mytilus*  
499 *edulis* contaminated with heavy metals. On this basis, the results presented in this study  
500 suggest that fish survival would be impaired after exposure to MD or CD, but impairment  
501 could be described as relatively less important following exposure to WSF, since the CAT  
502 activity was found to be significantly lower.

503 Exposure to the dispersant also seems to have induced toxicological effects, indicated by  
504 increases in SOD and GPx activities, even though the activities of these antioxidant enzymes  
505 did not reach those levels observed after CD, MD or WSF exposure. This result agreed with a  
506 recent study of Luna-Acosta *et al.* (2011) which revealed a modulation of SOD activity in the  
507 plasma of oysters, *Crassostrea gigas*, following dispersant exposure. This study and the  
508 present one exposed a modulation of biomarkers and thus highlighted the intrinsic toxicity of  
509 dispersant which disagreed with the statement of the manufacturer who claimed that  
510 dispersant is non-toxic at the concentration recommended by the manufacturer. This  
511 discrepancy could be due to the method used to determine the non-toxicity of the dispersant  
512 (NF.T.90-349). Indeed, this standard toxicity test measures the mortality rate of *Palaemonetes*  
513 *varians* exposed to several concentrations of a dispersant and compares them to the mortality  
514 rate observed for a reference toxic compound (N-alkyl dimethyl benzyl ammonium chloride).  
515 However, this toxicity test does not take into account sublethal effects of dispersants. Such

516 studies could be of interest in order to better estimate the toxicity levels of individual  
517 dispersants.

518 The antioxidant response suggested the production of ROS, which could potentially result in  
519 structural and functional impairments of the heart (Van der Oost, 2003). Thus, in order to  
520 better estimate the effects of the exposure treatments, it would be of interest to measure  
521 changes in integrity at the cellular and organ levels. For instance, at the cellular level, a comet  
522 assay to determine DNA integrity or an MDA (malondialdehyde, a product of oxidative lipid  
523 degradation) assay to evaluate lipid peroxidation could be conducted. At the organ level,  
524 cardiac performance could also be measured, since it has been suggested that this biological  
525 parameter is altered by oxidative stress (Luo *et al.*, 2006). A recent study of Milinkovitch *et*  
526 *al.* (2012) already investigated the force and the velocity of contraction in golden grey mullets  
527 exposed to similar treatments (WSF, D, MD, CD); results showed an impairment of the  
528 velocity of contraction for all treatments while no effect on the force of contraction. Similarly,  
529 cardiac abnormalities and defects in function could be studied, since Incardona *et al.* (2004)  
530 have already demonstrated a modification of heart morphology in PAH contaminated fish  
531 embryos (*Danio rerio*).

532 When compared to a previous and similar study (Milinkovitch *et al.*, 2011a), the present  
533 results showed that the biomarker responses were more marked in fish hearts than in other  
534 target organs, such as the liver. Indeed, in the previous study, after the same exposure  
535 treatments (CD, MD, WSF or D), no increases in antioxidant enzyme activities in fish livers  
536 were recorded. This is in accordance with Thomaz *et al.* (2009), who assessed antioxidant  
537 enzyme activities in several organs and who also suggested that the heart was more sensitive  
538 to the organophosphate insecticide trichlorfon than the liver or the gills. Taken together, these  
539 studies suggest that the heart could be considered as a target organ of interest for future

540 ecotoxicological studies. This finding agree with a recent study of Milinkovitch et al. (2012)  
541 who showed an impairment of cardiac performance in fish exposed to

542

## 543 **5. Conclusions**

544

545 The results of this study showed biological responses for all of the biomarkers tested (GSht,  
546 GST, GPx, SOD and CAT) and therefore indicated that the heart could be a target organ of  
547 interest when examining antioxidant responses and detoxification processes due to exposure  
548 to organic contaminants.

549 Most of the biomarkers tested (GSht, GPx, SOD) showed a modulation after exposure to a  
550 single dispersant (D), suggested this product has a sublethal toxicity. This disagrees with the  
551 acceptance of this ‘third generation’ dispersant as a non-toxic product. Thus, further studies  
552 are required to better estimate its intrinsic toxicity on pelagic organisms.

553 This study showed significant biomarkers responses after exposure to CD or MD, therefore  
554 suggesting that dispersed oil has toxicological effects. Such biomarkers responses have also  
555 been observed following WSF exposure for the biomarkers GSht, GST, SOD and GPx.  
556 However, results showed a significantly lower increase in CAT activity for WSF exposure  
557 (compared to MD and CD exposure) which could suggest that an undispersed oil slick would  
558 be less toxic to organisms living in the water column than a dispersed one. However, such an  
559 interpretation is based on a single biomarker response (on the 5 analysed) which incite us to  
560 be cautious. Further studies, for instance at other biological levels, would be recommended in  
561 the aim of highlighting the difference of toxicity that could exist between WSF and either CD  
562 and MD exposure.

563 Most of the biomarkers (GSht, GST, CAT and GPx) did not return to the control levels after  
564 14 days of depuration following the exposure of fish to oil (WSF, MD or CD). Thus, in



565 addition to the acute toxicity, oil and dispersed oil induced a toxicity that was still present  
566 after the depuration period. The assessment of biomarkers responses on a longer depuration  
567 period could provide further information concerning the long-term toxicity of hydrocarbons.  
568 No significant differences were observed between exposure to CD or MD. The difference  
569 between these two treatments is the addition of dispersant for CD exposure. Thus,  
570 extrapolating to the issues involved in oil spill response techniques, these results suggest that  
571 when environmental conditions (e.g. waves, wind, swell and tide) already provoke the natural  
572 dispersion of the oil slick (simulated by MD exposure), the addition of the dispersant (CD  
573 exposure) would not increase the environmental effects of oil. On the other hand, the use of  
574 dispersant would reduce the environmental cost of the oil spill by inhibiting the environmental  
575 risks associated with the re-coalescence of the oil slick (e.g. contamination of sea surface  
576 dwelling organisms, or drifting of the oil slick into ecologically relevant shoreline habitats)  
577 and by enhancing the bacterial degradation of petroleum.  
578 However, these conclusions should be considered cautiously, since they are based on the  
579 study of one species (*Liza aurata*) in a single compartment (water column). Consequently, the  
580 DISCOBIOL project (Dispersant et techniques de lutte en milieu côtier : effets biologiques et  
581 apport à la réglementation) intends to perform further studies focussing on several species  
582 from coastal areas (*Crassostera gigas*, *Mytilus edulis*, *Scophthalmus maximus*, *Dicentrarchus*  
583 *labrax* and *Liza aurata*) and examining the sediment as another compartment of interest.

584

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586

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