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Abstract

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

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

1. Introduction

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

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

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

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

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

2.3. Experimental system

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

2.4. Exposure treatments

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

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

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

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

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

2.5. Measurement of biomarker responses in fish hearts

The hearts were ground in a phosphate buffer (100 mM, pH 7.8) containing 20% glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a serine protease inhibitor. The homogenates were then centrifuged at 10,000 *g* and 4°C for 15 minutes to obtain the post-mitochondrial fraction (PMF). Total protein concentrations were determined using the method of Bradford (1992), with bovine serum albumin (Sigma-Aldrich Chemicals, France) as the standard. Prior to the biomarker response measurements, all assays were optimised for juvenile *Liza aurata* heart 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 µl 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 µmol 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 µg 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 µM) oxidation by 11 µg 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 $MnCl_2$. 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 µg 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 µg 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.

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253 2.6. Statistical analysis

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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 α=0.05. The results were expressed as means ± 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 ± 0.2 µmol 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

following dispersant (D) exposure was significantly lower than that of the control, but significantly higher than GSHt levels assessed after WSF, MD or CD exposures.

Following the 14 day depuration period (**B**), GSHt levels were not significantly different from those observed immediately after exposure (**A**). Concentration patterns also remained unchanged: the hearts of fish exposed to either WSF, MD or CD showed lower concentrations of glutathione and the hearts of fish exposed to the dispersant (D) showed significantly lower levels of glutathione when compared to those exposed to the control treatment, but higher levels than those exposed to WSF, MD or CD.

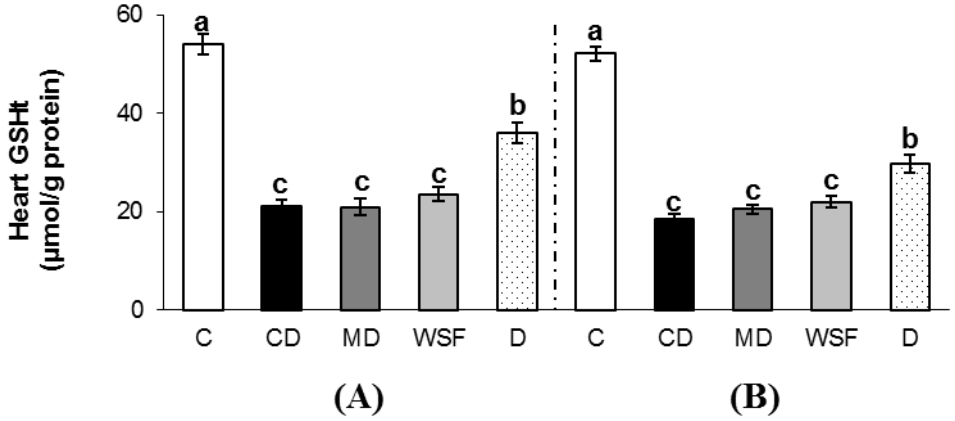

Figure 1: Concentration of total glutathione (GSHt) in the heart of *Liza aurata*. Glutathione concentration was measured after 48 h exposure **(A)** to Control (C), Chemically Dispersed oil (CD), Mechanically Dispersed oil (MD), Water Soluble Fraction (WSF) and Dispersant (D) exposure treatments; and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

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

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

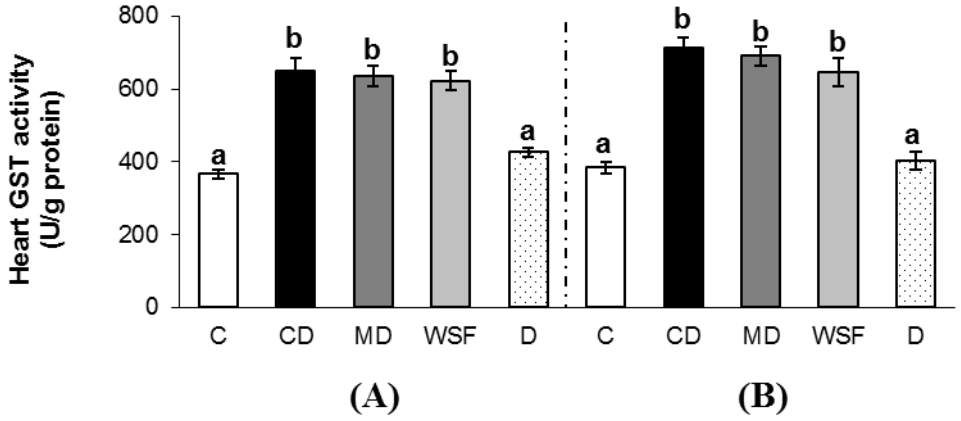

Figure 2: Glutathione-S-Transferase (GST) activity in the heart of *Liza aurata.* Activity was measured after 48 h exposure **(A)** to Control (C), Chemically Dispersed oil (CD), Mechanically Dispersed oil (MD), Water Soluble Fraction (WSF) and Dispersant (D) exposure treatments; and after 14 days of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

3.3. Superoxide dismutase (SOD) activity in fish hearts

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

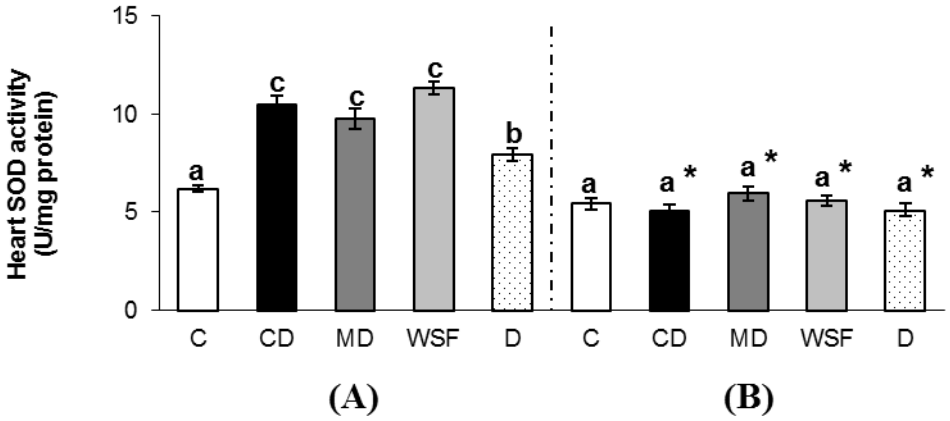

Figure 3: SuperOxide Dismutase (SOD) activity in the heart of *Liza aurata.* Activity was measured after 48 h exposure **(A)** to Control (C), Chemically Dispersed oil (CD), Mechanically Dispersed oil (MD), Water Soluble Fraction (WSF) and Dispersant (D) exposure treatments; and after 14 days of depuration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

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350 3.4. Catalase (CAT) activity in fish hearts

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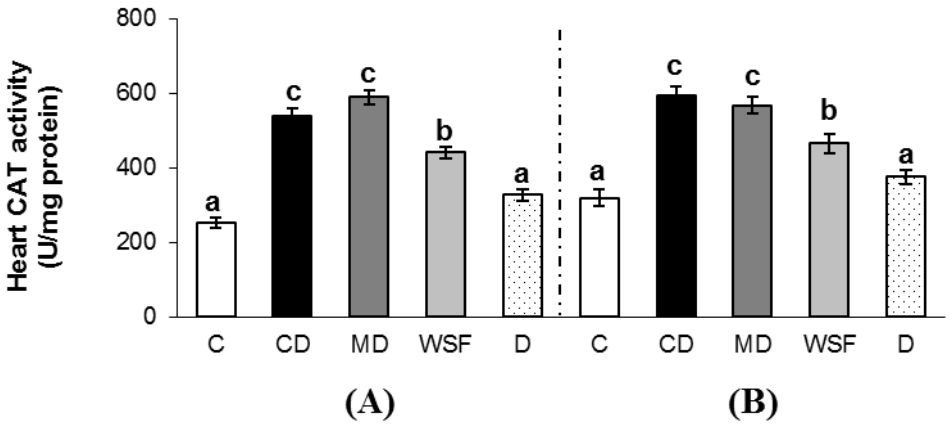
352 CAT activity (Figure 4) in the hearts of the control (C) *Liza aurata* was 253.3 ± 13.3 U/mg of
353 protein at the end of the 48 hour exposure period (**A**). Our results showed that the activity of
354 this enzyme was significantly increased by WSF, MD and CD exposures. The increase was
355 significantly 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)**

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

3.5. Glutathione peroxidase (GPx) activity in fish hearts

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

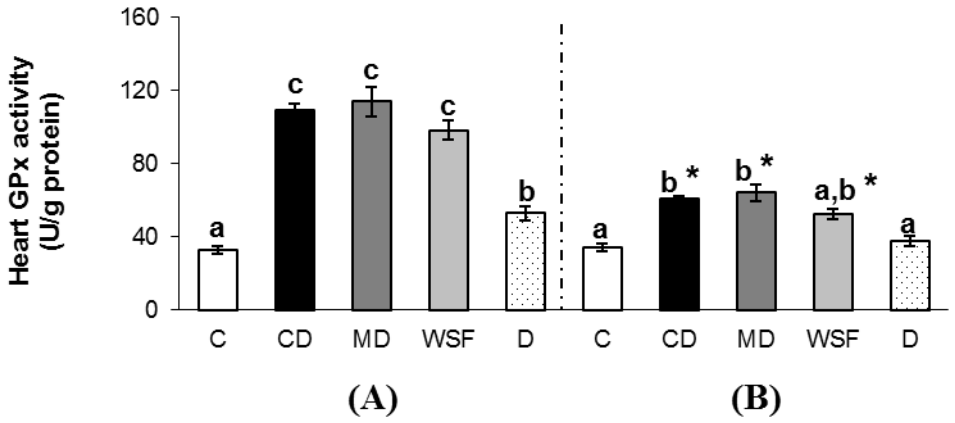


Figure 5: Glutathion peroxidase (GPx) activity in the heart of *Liza aurata.* Activity was measured after 48 h exposure **(A)** to Control (C), Chemically Dispersed oil (CD), Mechanically Dispersed oil (MD), Water Soluble Fraction (WSF) and Dispersant (D) exposure treatments; and after 14 days of depuration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

4. Discussion

4.1. Total glutathione content and GST activity in fish hearts

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

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

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

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

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

Comparing the biological effects with the seawater PAH concentrations, this study showed same magnitude of response in the GST activity levels between WSF and either CD and MD treatments while total concentrations of PAHs in CD and MD treatments are both approximately 8 and 50 times higher than in WSF, at the beginning (T0=0 h) and at the end of the exposure (T1=48 h) respectively. This could be due to the fact that GST activity in heart reached its maximum with WSF treatment and that consequently a higher load of contamination (through CD and MD exposure) did not induce a further increase of GST activity. Since GST activity is involved in the depletion of total glutathione, the same magnitude of response of this enzyme for WSF, CD and MD could explain the identical GSHt depletion observed for these three treatments.

4.2. Activity of antioxidant enzymes in fish hearts

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

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

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

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

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

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

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

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

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

5. Conclusions

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

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

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

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

addition to the acute toxicity, oil and dispersed oil induced a toxicity that was still present after the depuration period. The assessment of biomarkers responses on a longer depuration period could provide further information concerning the long-term toxicity of hydrocarbons.

No significant differences were observed between exposure to CD or MD. The difference between these two treatments is the addition of dispersant for CD exposure. Thus, extrapolating to the issues involved in oil spill response techniques, these results suggest that when environmental conditions (e.g. waves, wind, swell and tide) already provoke the natural dispersion of the oil slick (simulated by MD exposure), the addition of the dispersant (CD exposure) would not increase the environmental effects of oil. On the other hand, the use of dispersant would reduce the environmental cost of the oil spill by inhibiting the environmental risks associated with the recoalescence of the oil slick (e.g. contamination of sea surface dwelling organisms, or drifting of the oil slick into ecologically relevant shoreline habitats) and by enhancing the bacterial degradation of petroleum.

However, these conclusions should be considered cautiously, since they are based on the study of one species (*Liza aurata*) in a single compartment (water column). Consequently, the DISCOBIOL project (Dispersant et techniques de lutte en milieu côtier : effets biologiques et apport à la réglementation) intends to perform further studies focussing on several species from coastal areas (*Crassostera gigas, Mytilus edulis, Scophtalmus maximus, Dicentrarchus labrax* and *Liza aurata*) and examining the sediment as another compartment of interest.

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