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Abstract

Dispersant use in nearshore areas is likely to increase the exposure of aquatic organisms to petroleum. To measure the toxicity of this controversial response technique, golden grey mullets (*Liza aurata*) were exposed to mechanically dispersed oil, chemically dispersed oil, dispersant alone in seawater, water-soluble fraction of oil and to seawater as a control treatment. Several biomarkers were assessed in the gills (enzymatic antioxidant activities, glutathione content, lipid peroxidation) and in the gallbladder (polycylic aromatic hydrocarbons metabolites). The significant differences between chemically dispersed oil and water soluble fraction of oil highlight the environmental risk to disperse an oil slick when containment and recovery can be conducted. The lack of significance between chemically and mechanically dispersed oil suggests that dispersant application is no more toxic than the natural dispersion of the oil slick. The results of this study are of interest in order to establish dispersant use policies in nearshore areas.

Capsule:

When the meteorological conditions induce the dispersion of the oil slick (e.g. wave), the application of dispersant does not increase the toxicity of petroleum.

Keywords: dispersed crude oil; oxidative stress; glutathione; PAH biliary metabolites; gills; *Liza aurata*.

1. Introduction

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

77 Holdway, 2000; Ramachandran et al., 2004; Perkins et al., 2005; Jung et al., 2009), not taking
78 into account the presence of oil droplets in the water column. However, many field
79 observations have shown the presence of oil droplets in the water column. Their formation
80 can be induced within 2 hours (Cormack, 1977) or during a period of more than 1 week, as
81 observed during the Braer oil spill (Lunel, 1995). In this context, our experimental approach
82 was conducted in order to evaluate the actual toxicity of a chemically dispersed oil treatment
83 containing oil droplets. Toxicity was measured through the assessment of biomarkers in a
84 target organ of a pelagic fish species.
85 Oxidative stress and antioxidant defences were considered as suitable biomarkers since they
86 have been shown in many studies to respond to petroleum contamination and especially to the
87 PAH (polycyclic aromatic hydrocarbons) contained in petroleum (Avci et al., 2005 ; Almroth
88 et al., 2008; Oliveira et al., 2008; Jung et al., 2009; Kopecka Pylarczyk and Correia, 2009;
89 Narghang et al., 2009). Moreover, both, oxidative stress and antioxidant defences could give
90 information on the health of the contaminated organisms: (i) oxidative stress, since it is
91 considered as a cause of tissue injury (Halliwell, 1999) and (ii) antioxidant defences, since
92 authors linked modulation of this biological parameter to fish health indicators such as
93 progression of diseases and/or cellular mortality (Allen and Moore, 2004).
94 In our study, oxidative stress and antioxidant defences were assessed by evaluating lipid
95 peroxidation (a marker of lipid degradation due to oxidative stress) and evaluating the
96 response of antioxidant enzymatic activities (catalase, superoxide dismutase and glutathione
97 peroxidase), respectively.
98 Additionally, total glutathione was measured taking into account the importance of the
99 cellular status of this molecule for the defence of the organism against xenobiotics (Maracine
100 and Segner, 1998). Indeed, glutathione is implied in many cellular defence mechanisms such
101 as (i) antioxidant defences, by its conjugation to reactive oxygen species (Amiard-Triquet and

Amiard, 2008); (ii) heavy metals (such as Vanadium and Nickel present in petroleum, Salar Amoli et al., 2006) chelation, as described in Sies, 1999; and (iii) detoxification processes, by its conjugation to xenobiotics such as PAH (van der Oost et al., 2003). In our study, we evaluated the glutathione status through the measurement of the total glutathione content which is the sum of the oxidized and the reduced form of this molecule.

These biomarkers were assessed in fish gills, taking into account their target organ status: several studies have shown an effect of petroleum compounds on gills (McKeown, 1981; Oliveira et al., 2008; Mendonça Duarte et al., 2010). In parallel, PAH biliary metabolites were measured in order to evaluate the level of exposure to PAH following dispersant application.

The choice of the golden grey mullet (*Liza aurata*) as a biological model was based on the fact that (i) it represents a relevant biomass in nearshore ecosystems; (ii) it is a commercially important species especially in Europe and Egypt (Gautier and Hussenot, 2005) and (iii) this species is present in nearshore areas during its early life stages (Gautier and Hussenot, 2005) being consequently a target organism for anthropogenic pollutants (Bruslé, 1981).

2. Materials and methods

2.1. Experimental design

2.1.1. Experimental animals

Fifty juvenile golden grey mullets (*Liza aurata*), fished in Venice (Italy) lagoons and provided by Commercio Pesca Novellame Srl (Chioggia, Italy), were used to conduct this study.

For 4 weeks, fish were acclimatized in 300-L flow-through tanks prior to the exposure studies (dissolved oxygen: 94 ± 2%; salinity: 35 ± 0%; temperature: 14.9 ± 0.5 °C, with a 12 h light:12 h dark photoperiod in seawater free of nitrate and nitrite). During acclimation, they were fed daily with fish food (Neosupra AL3, from Le Gouessant aquaculture) which does not contain additives (also called synthetics) antioxidants authorised by the European Union (butyl-hydroxy-anisol, butyl-hydroxy-toluene, ethoxyquin, propyl gallate and octyl gallate). Fish were starved for 48 h prior to bioassays and throughout the exposure period, in order to avoid bile evacuation from the gallbladder. Prior to bioassay, their average length was 136.6 ± 0.1 mm (mean ± standard error of the mean) and their average weight was 32.33 ± 0.87 g.

2.1.2 Chemicals

A dispersant formulation (Total Fluides) was selected based on its efficiency. The efficiency was preliminary determined in the CEDRE (CEntre de Documentation de Recherche et d'Expérimentations sur les pollutions accidentelles des eaux, France) using the method NF.T.90-345. The dispersant was non-toxic at the concentration recommended by the manufacturer (preliminary determined using standard toxicity test: method NF.T.90-349) and biodegradable.

A Brut Arabian Light (BAL) crude oil was selected for this study. The oil is composed of 54% saturated hydrocarbons, 10% polar compounds and 36% aromatic hydrocarbons.

To simulate the natural behaviour of the oil after it is released at sea (evaporation of light compounds and natural photodegradation, respectively) the oil was evaporated under atmospheric conditions and natural UV-sunlight, prior to fish exposure. The resulting chemical composition of the oil was 54% saturated hydrocarbons, 12% polar compounds and 34% aromatic hydrocarbons. Among aromatic hydrocarbons, concentration of 21 PAH was

measured (the 16 PAH listed by the USEPA as priority pollutants and five supplementary PAH: benzo[*b*]thiophene, biphenyl, dibenzothiophene, benzo[*e*]pyrene, perylene). The sum of the 21 PAH represents 16.4 mg/g of petroleum (1.64 % of the petroleum). More information concerning the composition of the petroleum used in this study is available in (Milinkovitch et al., accepted for publication).

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

The experimental system (also described in Milinkovitch et al., 2011) comprised five 300-L seawater tanks. Each one contains a funnel (**a,** at the surface) linked to a Johnson L450 water pump (**b**, at the bottom of the tank). After 24 h homogenization, this system was set up to maintain a mixture of oil and dispersant as a homogenous solution despite the hydrophobic character of the oil (preliminary tests not shown). The temperature in this static water system was controlled using two heaters (RENA CAL 300) so that the exposure temperature was 15.3 ± 0.3 °C (mean ± standard error mean). Other physico-chemical parameters were also measured: seawater was free of nitrate and nitrite, pH (7.99 ± 0.03) and dissolved oxygen (98 ± 5%) remained constant throughout the study.

2.1.4. Experimental treatments

Each experimental tank contained 300 L seawater provided by Oceanopolis (France). The control treatment (C) was made up using 300 L seawater. The chemically dispersed (CD) oil treatment was made by pouring 20 g of petroleum and 1 g of dispersant into the funnel of the experimental system. The mechanically dispersed (MD) oil treatment was made by pouring 20 g of petroleum into this funnel. The dispersant alone (D) treatment, as an internal control

of CD, was made by pouring 1 g of dispersant into the funnel. For the water-soluble fraction of oil (WSF), a 20 g oil slick was contained using a plastic cylinder (21 cm diameter) placed on the surface of the seawater (in addition to the funnel and the pump, which were kept to maintain the same level of agitation of the seawater as for other treatments). Readers must take into account that the spreading of the oil slick was not prevented by the plastic cylinder since the oil slick was smaller than the diameter of the plastic cylinder. Thereby the experimental approach simulates the actual spreading behaviour of oil at sea. During the entire exposure period, the oil slick remained at the surface without mixing. No droplet was observed in the water column (visual observations) suggesting that the fish were only exposed to the soluble fraction of the oil.

While the solutions remained homogenous (less than 5 % difference between three TPH concentration measurements sampled at three differents depths in the experimental tanks), no fish were exposed for 24 hours after making up the solutions. Then, groups of 10 fish were randomly distributed in the five experimental tanks, each tank containing an exposure media (described above). The fish were exposed for 48 h (from T=0 h to T=48 h).

At the end of the exposure period, the fish in each tank (each treatment) were euthanized using eugenol (99 %, Sigma Aldricht chemicals, France). The gallbladder was removed from each fish and stored at –80 °C prior to analysis. Gills were rinsed off by dipping them in PBS (Phosphate Buffered Sodium 0.01 M, pH=7.4, Sigma) in order to remove blood. Then, the gills were homogenized in another PBS solution. The homogenates were centrifuged at 10,000 g, 4 °C, for 15 min to obtain the post-mitochondrial supernatant. Total protein concentrations in supernatants were determined using the method of Bradford (1976) with bovine serum albumin (Sigma-Aldrich Chemicals, France). Then, supernatants were stored at –80 °C prior to biochemical analysis.

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202 2.2. Total petroleum hydrocarbon (TPH) seawater concentrations

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204 The TPH concentration, which is the sum of the dissolved hydrocarbon concentrations plus
205 the amount of oil droplets, was measured for all treatments at the beginning (T=0 h) and at the
206 end of fish exposure (T=48 h), using the mean of three replicated measurements for each time
207 point. The samples were extracted with 10 mL of dichloromethane (Carlo Erba Reactifs,
208 SDS). After separation of the organic and aqueous phases, water was extracted two additional
209 times with the same volume of dichloromethane (2 x 10 mL). The combined extracts were
210 dried on anhydrous sulphate and then analysed using a UV spectrophotometer (UV-Vis
211 spectrophometer, Unicam, France) at 390 nm, as described by Fusey and Oudot (1976).
212 Assays were conducted in collaboration with Cedre (Centre de Documentation de Recherche
213 et d'Expérimentations sur les Pollutions Accidentelles des Eaux), a laboratory with agreement
214 ISO 9001 and ISO 14001. In accordance with Cedre, results are not reliable under 1mg/L.

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216 2.3. Biochemical analysis

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218 2.3.1. Fixed wavelength fluorescence analysis of bile

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220 Bile samples were diluted (1:1000) in absolute ethanol (VWR International, France) and
221 assessments were conducted for three fixed wavelength fluorescence (FF). FF 290:335 mainly
222 detects naphthalene-derived metabolites, FF 341:383 mainly detects pyrene-derived
223 metabolites and FF 380:430 mainly detects benzo[*a*]pyrene-derived metabolites (Aas et al.,
224 2000). Measurements were performed in quartz cuvettes (Sigma Aldricht, USA) on a
225 spectrofluorimeter (SAFAS Flx-Xenius, Monaco). The FF values were expressed as arbitrary

units of fluorescence and the signal level of pure ethanol was subtracted. Depending on the spectrofluorimeter, results are not reliable under 0.1 arbitrary units.

2.3.2. Total glutathione (GSH)

Total (reduced plus oxidized) glutathione was determined spectrophotometrically in gills, according to the procedure of Akerboom and Sies (1981) and using a glutathione assay kit (SIGMA CS0260, Sigma Aldricht, USA). The samples were first deproteinized with 5% 5-sulfosalicylic acid solution. The glutathione content of the sample was then assayed using a kinetic assay in which amounts of glutathione cause a continuous reduction of 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) to TNB. The oxidized glutathione formed was recycled by glutathione reductase and NADPH. The product, TNB, was assayed colorimetrically at 412 nm in UV microplates (Greiner Bio One), using a spectrophotometer (SAFAS Flx-Xenius, Monaco). The results are presented in µmol of GSH/g of protein.

2.3.3. Antioxidant enzymes and lipid peroxidation

Glutathione peroxidase (GPx) activity was determined according to the method of Paglia and Valentine (1967), using a glutathione peroxidase assay kit (RS504/RS 505, RANDOX, France). GPx catalyses the oxidation of reduced glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH the oxidized glutathione (GSSG) is immediately converted to the reduced form with concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance was measured at 340 nm.

Superoxide dismutase (SOD) activity was determined according to the method of Wooliams et al. (1983) and using a superoxide dismutase assay kit (SD125, RANDOX, France). This

method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye, assessed at 505 nm. The superoxide dismutase activity was then measured by the degree of inhibition of this reaction. One unit of SOD was that which causes a 50% inhibition of the rate of reduction of INT. The results are presented in units of SOD/mg of protein.

Catalase (CAT) activity was determined according to the method of Deisseroth and Dounce (1970) and using a catalase assay kit (CAT 100, Sigma Aldricht, USA). Samples were mixed (v:v) with hydrogen peroxide. The kinetics of hydrogen peroxide degradation were assessed at 280 nm. The results are expressed in units of CAT/mg of protein.

Lipid peroxidation levels were assessed via malondialdehyde (MDA) contents determined using a commercially available MDA assay kit (MDA assay kit, Oxis International, USA). The method was based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA. The blue product was quantified by measuring absorbance at 586 nm (Gérard-Monnier et al., 1998). The results are presented in nmol of MDA/g of tissue.

Materials used to measure spectrophotometrically antioxidant enzymes activity and lipid peroxidation were UV microplates (Greiner Bio One, Germany) and a spectrophotometer (SAFAS Flx-Xenius, Monaco).

2.4. Statistical analysis

The statistical analysis was carried out using XLstat 2007 software. The assumptions of normality and homoscedasticity were verified using the Kolmogorov-Smirnov and Cochran tests, respectively. When homoscedasticity and normality were not respected, a Kruskal Wallis test was conducted to highlight significant differences between treatments. When

homoscedasticity and normality were respected, a factorial analysis of variance (one-way ANOVA) was performed in order to assess the effects of the different treatments. This statistical analysis was followed by the Tukey post-hoc test to detect significant differences between groups. Correlations between fixed wavelength fluorescence intensity and other variables (GSH, GPx, SOD, CAT and LPO) were conducted using the Spearman test. The significance of the results was ascertained at $\alpha=0.05$. The results are expressed as means ± s.e.m. (standard error of the mean) corresponding to groups of 10 fish (n=10).

3. Results

No fish died during the acclimation and exposure period. TPH were not detected in the Control (C) and Dispersant (D) treatments. Moreover no oil slick was observed in the Chemically Dispersed oil (CD) and the Mechanically Dispersed oil (MD) treatments. In the Water Soluble Fraction of oil (WSF) treatment, the oil slick remained at the surface throughout the exposure period and no droplets were observed (visual observations) in the water column.

3.1. Total petroleum hydrocarbons (TPH)

The concentration of TPH (**Table 1**) was slightly higher in the CD than in the MD treatment at T=0 h and at T=48 h. A 68% decrease was observed in the CD treatment (from 46.4 to 14.9 mg/L) and a 73% decrease was observed in the MD treatment (from 39.4 to 10.7 mg/L) during the 48 h exposure period. No TPH were detected in the WSF treatment, probably due to the detection limit of the method.

3.2. Fixed wavelength fluorescence analysis of bile

Whatever the fixed wavelength employed (**Figure 2**), no significant difference was found between the fluorescence intensity of the WSF, D and C treatments.

Whatever the fixed wavelength employed, the fluorescence intensity was significantly higher in the CD treatment than in the C, D and WSF treatments.

At FF 380:430 and FF 343:383, the fluorescence intensity was higher in the MD treatment than in the C, D and WSF treatments whereas no significant difference was found at FF 290:335.

At FF 290:335 and FF 343:383, the fluorescence intensity was lower in the MD treatment than in the CD treatment whereas no significant difference was observed at FF 380:430.

3.3. Total glutathione (GSH)

Gill GSH content (**Figure 3**) was significantly lower in the CD than in the C, D and WSF treatments, whereas no significant difference was observed between the CD and MD treatments. No significant difference was observed between MD and the other treatments (C, D and WSF).

Significant correlations were found between the fluorescence intensities FF 343:383 and FF 380:430 with GSH content ($P= 0.001$ and $P=0.002$ respectively) whereas there was no correlation between the fluorescence intensity at 290:335 with GSH content ($P>0.05$).

3.4. Antioxidant enzymatic activity and lipid peroxidation (LPO)

No significant difference was found between the five treatments ($P>0.05$), in terms of antioxidant enzymatic (SOD, CAT, GPx) activities (**Figure 4**). With regards to SOD activity, the lack of significance could be due to the high intragroup variability. With regards to GPx, the enzymatic activity seemed to be higher in the CD treatment than in the other treatments. No correlation was found between the enzymatic activities and fixed wavelength fluorescence intensity ($P>0.05$). There was no significant difference in LPO (**Figure 5**) between the five treatments ($P>0.05$) and no correlation was found between LPO and the fixed wavelength fluorescence intensities ($P>0.05$).

4. Discussion

The aim of this study was to evaluate the toxicity of dispersant application. Through an experimental approach, several scenarios occurring during an oil spill were considered and their toxicity was evaluated. Five exposure treatments were conducted: (i) a control treatment with only seawater, (ii) a chemically dispersed oil treatment simulating, *in situ*, dispersant application on an oil slick under mixing processes, (iii) dispersant alone in seawater as an internal control of CD, (iv) a mechanically dispersed oil simulating only the effect of mixing processes on the oil slick and (v) a water-soluble fraction of oil simulating contamination due to an undispersed oil slick.

Given observations at oil spill sites (such as during the Braer oil spill, Lunel et al., 1995) and the natural mixing processes in nearshore areas (e.g. waves), the presence of oil droplets in the water column seems to be relevant when evaluating the toxicity of dispersant application in nearshore areas. Thus, the experimental system was devised to maintain oil droplets in the water column throughout the course of exposure.

351 4.1. Total petroleum hydrocarbons (TPH)

352

353 TPH concentrations vary from 46.4 to 14.9 mg/L for CD treatment and from 39.4 to 10.7
354 mg/L for MD treatment. The concentrations observed at T = 0 h are inferior to the nominal
355 concentrations (66.6 mg/L). This is probably due to the petroleum adherence to the
356 experimental system during the 24 h period of homogenisation (prior to the bioassays,
357 described in **2.1.5.**). The concentrations of TPH, measured in this experimental approach, are
358 consistent with those observed at oil spill sites. Indeed, Spooner (1970) observed 50 mg/L of
359 TPH after an oil spill in Tarut Bay (Saudi Arabia) due to a pipeline fracture. This observed
360 concentration was due to the natural dispersion of 16 000 t of light Arabian crude oil in
361 nearshore areas (less than 2 km from the shoreline). In the same way, Lunel (1995) observed
362 concentrations varying between 1 and 100 mg/L during the wreck of the Braer on the
363 Scotland coast. The cargo released 86 000 t of Gullfaks crude oil which were naturally
364 dispersed due to severe wind conditions (Force 6 to 10).
365 Braer oil spill shows that, in nearshore areas, meteorological conditions could induce
366 dispersion of the oil slick during a period of more than one week. However, at most oil spill
367 sites in offshore areas, a decrease in concentration is observed over a 2 to 5 h period (Lessard
368 and Demarco, 2000). Situated between these two scenarios, our experimental approach
369 showed a decrease in TPH concentration over a 48 h period. Our observations suggest that
370 this decrease is mainly due to petroleum adherence to the experimental system. This
371 phenomenon of adherence to the experimental system simulates the adherence to the substrate
372 observed in field studies (Baca et al., 2006). In this study, adhered petroleum represents
373 approximately the nominal concentration of the petroleum minus the concentration of
374 petroleum assessed in the water column. Even if adhered petroleum represents a relevant
375 proportion of the petroleum (in particular at T = 48 h), fish were not directly exposed to this

fraction of the petroleum since (i) pelagic fish species, such as golden grey mullets, should only be exposed to petroleum present in the water column; (ii) in our study, most of the adhered petroleum was present in the funnel, for which fish do not have access.

4.2. PAH biliary metabolites

The relative concentration of PAH biliary metabolites (evaluated through fixed wavelength fluorescence analysis) has often been used as an exposure biomarker (Camus et al., 1998; Aas et al., 2000; Jung et al., 2009). PAH are well studied since they are considered to be the most toxic compounds of petroleum. In our study we measured the biliary-derived metabolites corresponding to PAH (alkylated and parents) of three different weights (naphthalene: 128.2 $g.mol^{-1}$, pyrene: 202.3 $g.mol^{-1}$, benzo[*a*]pyrene: 252.3 $g.mol^{-1}$). The results showed a significant increase in the three PAH metabolites following the CD treatment, when compared to WSF. This result is in accordance with many studies (Perkins et al., 1973; Cohen and Nugegoda, 2000; Ramachandran et al., 2004; Lin et al., 2009) since it shows that the application of dispersant on an undispersed oil slick increases PAH exposure. The same is true of the MD treatment, when compared to WSF: mechanical dispersion increased pyrene and benzo[*a*]pyrene exposure (however no significant difference was observed for naphthalene-derived metabolites). This increase in PAH exposure, due to the dispersion (chemical or mechanical), suggests an increase of toxicity for tested organisms. Indeed, PAH are considered as carcinogenic and mutagenic (Eisler, 1987). Moreover, studies revealed that PAH induce histopathological effects (Stentiford et al., 2003 ; Ortiz-Delgado et al., 2007), inflammatory responses (Stentiford et al., 2003), oxidative stress (Sun et al., 2006 ; Oliveira et al., 2008) and alterations of DNA integrity (Oliveira et al., 2007 ; Maria et al., 2002) in teleost fish.

With regards to the MD and CD treatment, our results show that the differences in the relative concentration of the metabolites seem to be linked to PAH toxicity: the more toxic a PAH, the lower the difference, in metabolite concentration, between the two treatments. Indeed, naphthalene-derived metabolites (described as low toxicity PAH in Petry et al., 1996 and Bosveld et al., 2002) showed a 40% increase with CD treatment (when compared to MD treatment). Pyrene-derived metabolites showed a 13% increase. No significant difference was observed for benzo[*a*]pyrene-derived metabolites, which is considered as a carcinogenic PAH and induces reactive oxygen species (Lemaire-Gony and Lemaire, 1993).

4.3. Total glutathione content (GSH)

When compared to the WSF treatment, the CD treatment induced a significant decrease in total glutathione content in the gills. Several hypotheses may explain the decrease in GSH content, such as the conjugation of glutathione to PAH through the increase in GST activity as observed in Yin et al. (2007) or the decrease in GSH synthesis due to contaminant exposure as described in Canesi et al. (1999). Whatever the physiological mechanism implicated, this study shows that dispersant application induced a depletion of glutathione, which is the first line cellular defence involved in many detoxification processes (Maracine and Segner, 1998). Thereby, the chemical dispersion of an oil slick decreases the potential of fish to cope with contaminated environments.

On the contrary, when compared to the MD treatment, the CD treatment did not induce a significant decrease in the total glutathione content in the gills, suggesting that, even when the oil slick is mechanically dispersed (e.g. due to meteorological conditions), the application of dispersant does not significantly decrease the potential of the organism to cope with its environment.

Benzo[*a*]pyrene- and pyrene-derived metabolite concentrations were correlated with the total glutathione content in the gills. However no correlation was found between naphthalene-derived metabolites and total glutathione content. Taken together, these results suggest that glutathione depletion arises due to exposure to heavy PAH whereas light PAH would not be involved in the observed decrease in glutathione.

In Milinkovitch et al. (2011), a similar experimental approach was conducted with the same exposure treatments as described in this study (C, CD, MD, WSF, D). The total glutathione content in fish liver was evaluated and appeared to follow the same pattern as the total glutathione content in gills (exposed in this study): CD treatment induced a significant decrease of total glutathione when compared to control treatment; and no significant difference was observed between CD and MD treatments. However, no significant difference was observed concerning the liver total glutathione content between WSF and CD exposure whereas, in the present study, when studying the fish gills, a significant difference was observed between these both conditions. This finding shows that, evaluating dispersant application toxicity, gill seems to be a more sensitive target organ than liver. This relevant sensitivity of gills could be due to the fact that gills are target organs immediately in contact with the external environment and thereby immediately in contact with pollutants presents in the water column.

4.4. Oxidative stress

PAH, when incorporated by the organism, are bound to a cellular aryl hydrocarbon receptor (AhR). This binding induces the formation of a complex, the aryl hydrocarbon receptor nuclear translocator (ARNT), which is delocalized in the nucleus of the cell and bound to the xenobiotic regulatory element (XRE). This phenomenon increases the transcription rate of the

P4501A cytochrome genes (CYP1A) and by the way increases the synthesis *de novo* of the cytochrome P450 enzymes and the catalytic activity of these enzymes (Stegeman, 1987). This increasing activity enhances the cellular production of reactive oxygen species (Livingstone, 2001), which is counteracted by the antioxidant response (especially through enzymatic antioxidant activities). When the production of ROS overwhelms the antioxidant response, free reactive oxygen species can interact deleteriously with cellular components. Lipid peroxidation is a marker of this impairment.

Our results showed no modulation of lipid peroxidation, suggesting a lack of free radical attack due to PAH exposure. Moreover, no antioxidant response was observed. The absence of oxidative stress could be due to the composition of the fish food. Indeed, even if fish were fed during four weeks with a fish food free of additives (also called synthetics) antioxidants, natural antioxidants (such as vitamins A, C and E) are presents in the food composition. This consummation of antioxidants could have prevented fish against oxidative stress.

Another explanation concerning this lack of significance could also be due to the fact that the exposure period was too short to induce ROS production. Indeed, although some studies have shown some effects of PAH following a short exposure period (≤ 48 h, Sun et al., 2006; Oliveira et al., 2008), many studies investigated the effects of contaminants on oxidative stress by exposing animals to longer periods (Kopecka-Pilarczyk and Correia, 2009; Jung et al., 2009; Narghang et al., 2009; Hannam et al., 2010).

5. Conclusion

With regards to gill glutathione content and the relative concentration of PAH biliary metabolites, the results of this study firstly demonstrate that WSF exposure may be less toxic than CD exposure. These results are in accordance with the TPH concentrations measured in

476 sea water, suggesting, in accordance with the literature (Perkins et al., 1973; Cohen and
477 Nugegoda, 2000; Ramachandran et al., 2004; Lin et al., 2009), that the transfer of
478 hydrocarbons (from the sea surface to the water column) due to dispersant application led to
479 an increase of toxicity. Extrapolated to field operations, results of this study mean that
480 containment and recovery, rather than chemical dispersion of the oil slick, must be conducted.
481 However, depending on technical facilities and meteorological conditions, it is not always
482 possible to contain the oil slick. In some oil spill situations (e.g. rough sea and low viscosity
483 petroleum), dispersant is the only appropriated response technique.
484 Since a minimum sea energy is required before a dispersant functions effectively (Merlin,
485 2005) and since nearshore areas are considered to be turbulent zones (due to waves, wind and
486 swell) it seemed important, in this study, to evaluate the toxicity of dispersant application
487 under a mixing process. Comparison of MD and CD showed a significant difference
488 concerning low toxicity PAH-derived metabolites (naphthalene and pyrene) - these results are
489 in accordance with the TPH concentrations measured in sea water -. However, no significant
490 difference was found for benzo[*a*]pyrene-derived metabolites, which are considered to be
491 carcinogenic and to induce reactive oxygen species. Moreover no significant difference was
492 found between the glutathione content following the CD and MD treatments. Taken together,
493 these results suggest (i) no increase of toxic compounds exposure due to dispersant
494 application and (ii) no increase of organism detoxification response. These results suggest
495 that, when an oil slick is naturally dispersed, the application of dispersant seems to not
496 increase its environmental toxicity. These results are in accordance with a similar previous
497 study (Milinkovitch et al., 2011).
498 However, several limits of this experimental approach compel us to be cautious in our
499 conclusions. Indeed, the experimental approach is available only for a given turbulent mixing
500 energy (the energy induced by the experimental system). Moreover, this experimental

approach only takes into account the toxicity to pelagic teleost fish while other components of the ecosystem are also likely to be impaired by dispersant application. An experimental approach considering the environmental conditions and other components of an ecosystem (benthic and demersal species) would provide supplementary information. In this context, further studies as part of the DISCOBIOL project will evaluate the impact of dispersed oil on burrowing organisms, demersal organisms (such as oysters) and pelagic species (such as golden grey mullet) within an enclosed ecosystem (mesocosm).

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Table 1: TPH and dispersant nominal concentration in the five exposure media at the beginning (T=0 h) and at the end of the exposure (T=48 h) to C (Control), CD (Chemically Dispersed oil), MD (Mechanically Dispersed oil), WSF (Water Soluble Fraction of oil) and D (Dispersant).

Values are expressed as mean ± standard error mean of both experimental replicates. n.d. = not detected.

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

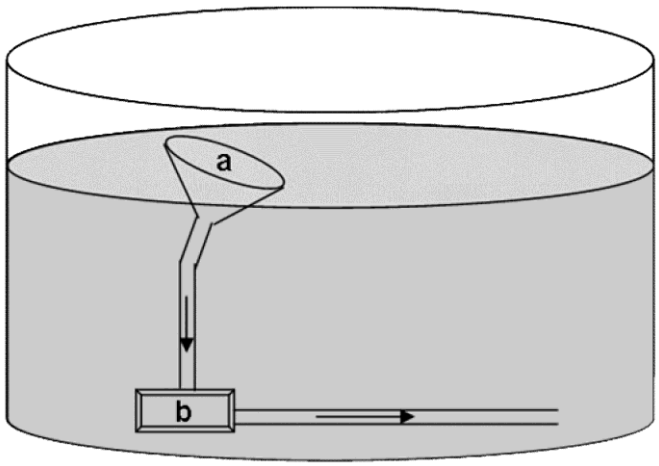


Figure 1: The experimental system constituted of a funnel (**a**) linked to a water pump (**b**) in a 300-l sea tank. → indicates the direction of seawater and/or contaminants through the experimental system

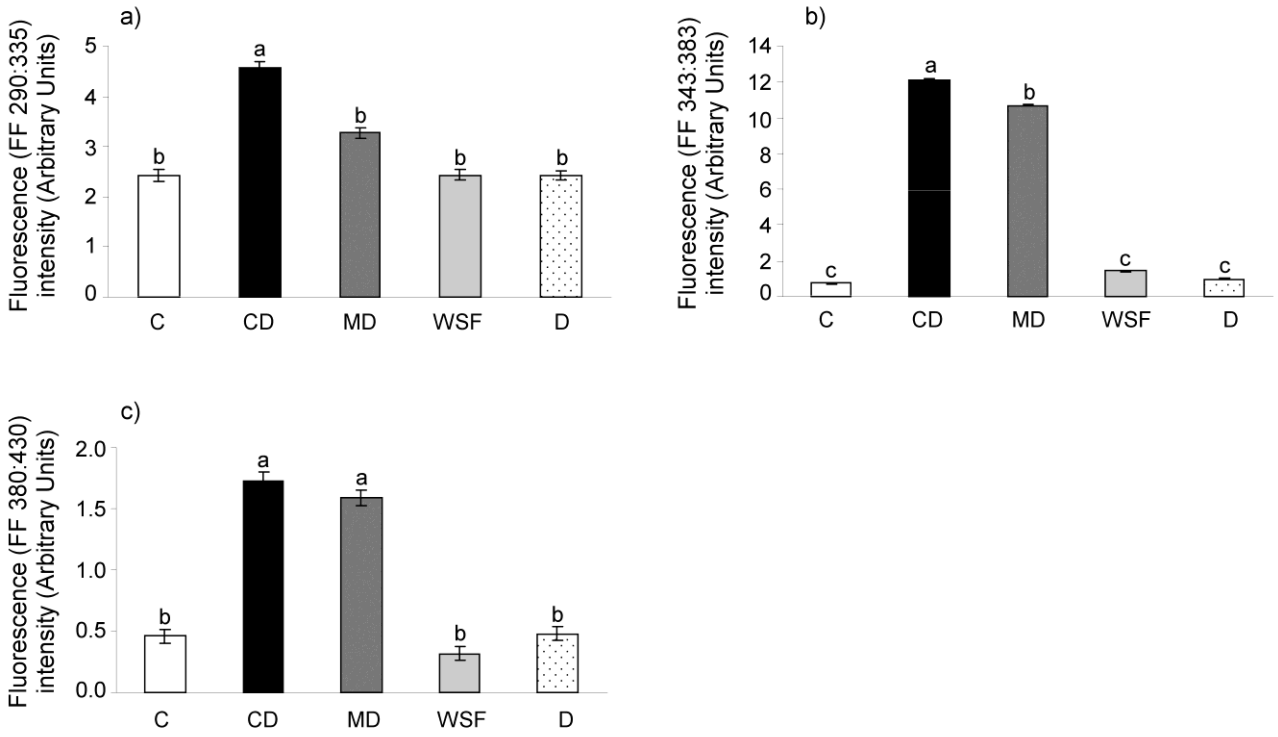


Figure 2: Concentration of biliary PAH metabolites measured by fixed wavelength fluorescence (FF) levels after 48 h exposure to Control solution (C), Chemically Dispersed oil solution (CD), Mechanically Dispersed oil solution (MD), Water Soluble Fraction (WSF) solution and Dispersant solution (D) : (a) FF 290:335 (naphthalene type derived metabolites); (b) FF 343:383 (pyrene derived type of metabolites); (c) FF 380:430 (benzo[*a*]pyrene type of metabolites). Levels are expressed as fluorescence intensity. Values represent mean ± standard error (n=10 per treatment). Different letters above bars indicate a significant difference**,** where $P < 0.05$.

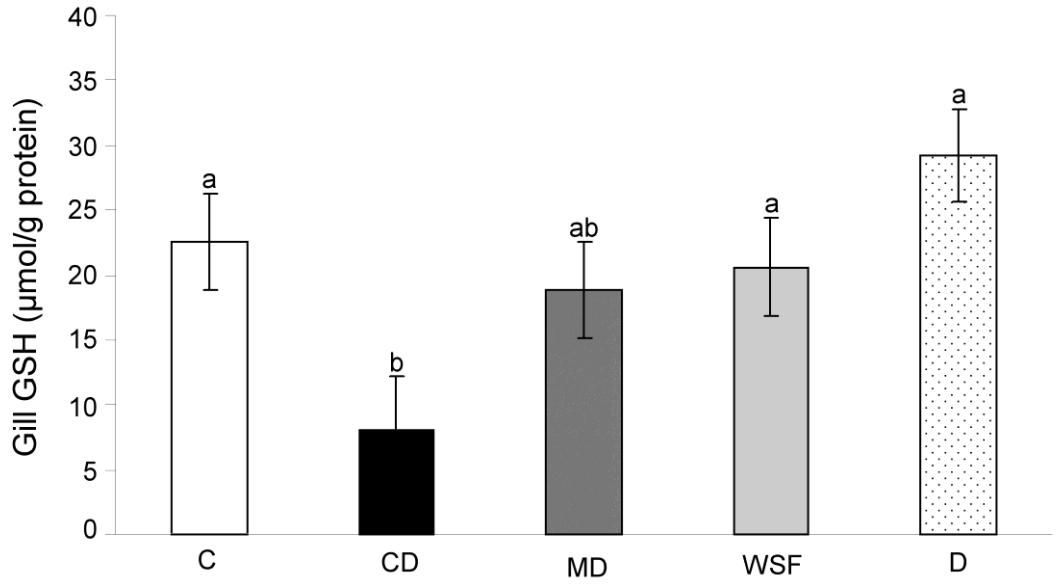
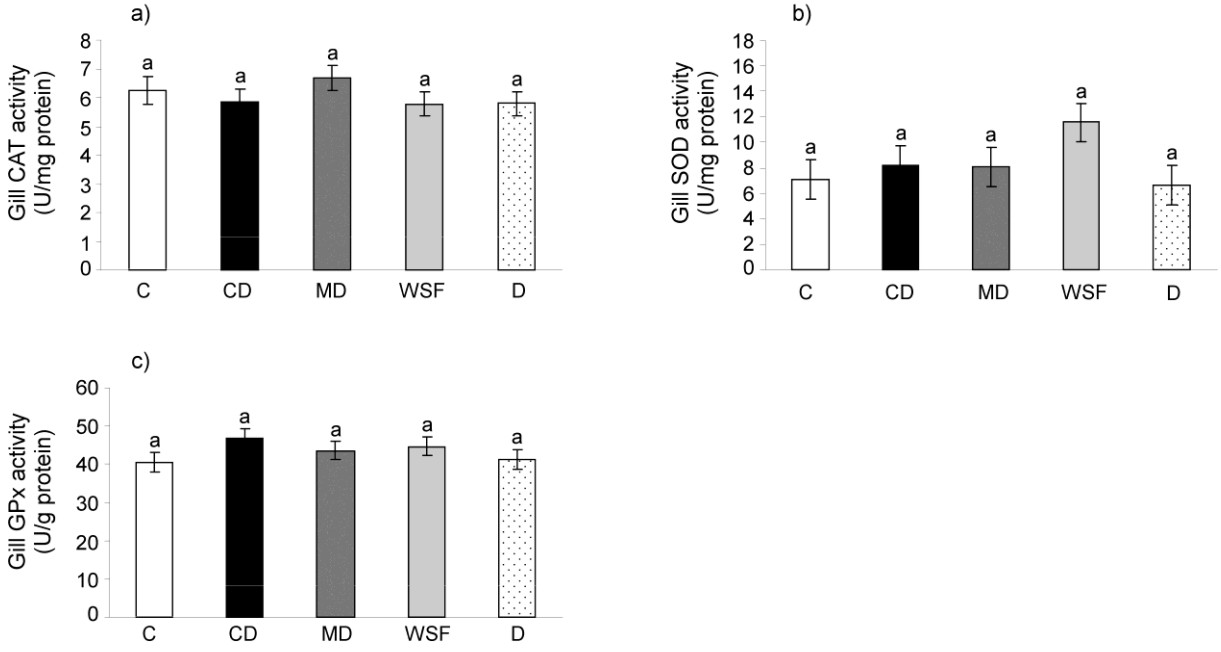


Figure 3: Total glutathione (GSH) content in gills of *Liza aurata* after 48 h exposure to Control solution (C), Chemically Dispersed oil solution (CD), Mechanically Dispersed oil solution (MD), Water Soluble Fraction (WSF) solution and Dispersant solution (D). Values represent mean ± standard error (n=10 per treatment). Different letters above bars indicate a significant difference**,** where $P < 0.05$.



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

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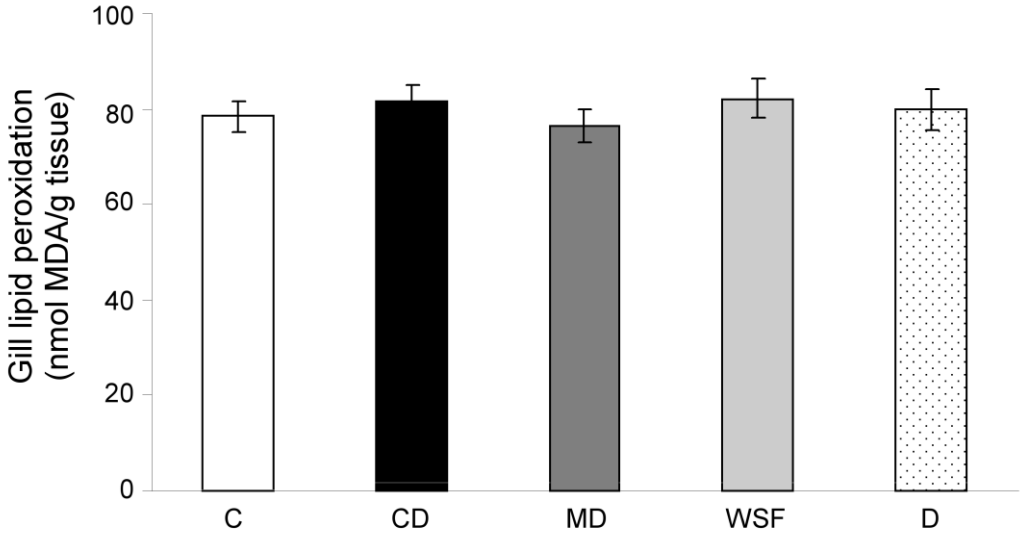

777

778 **Figure 5**: Lipid peroxidation in gills of *Liza aurata* after 48 h exposure to Control solution
779 (C), Chemically Dispersed oil solution (CD), Mechanically Dispersed oil solution (MD),
780 Water Soluble Fraction (WSF) solution and Dispersant solution (D). Values represent mean ±
781 standard error (n=10 per treatment). Different letters above bars indicate a significant
782 difference**,** where $P < 0.05$.

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