HAL
open science

Liver antioxidant and plasmatic immune responses in juvenile golden grey mullet (Liza aurata) exposed to dispersed crude oil

Thomas Milinkovitch, Awa Ndiaye, Wilfried Sanchez, Stéphane Le Floch, Hélène Thomas-Guyon

To cite this version:

Thomas Milinkovitch, Awa Ndiaye, Wilfried Sanchez, Stéphane Le Floch, Hélène Thomas-Guyon. Liver antioxidant and plasmatic immune responses in juvenile golden grey mullet (Liza aurata) exposed to dispersed crude oil. Aquatic Toxicology, 2011, 101 (1), pp.155-164. 10.1016/j.aquatox.2010.09.013 . hal-00647228

HAL Id: hal-00647228

https://hal.science/hal-00647228

Submitted on 1 Dec 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Liver antioxidant and plasmatic immune responses in juvenile golden grey mullet (*Liza aurata*) exposed to dispersed crude oil

Thomas Milinkovitch[1*], Awa Ndiaye[2], Wilfried Sanchez[2], Stéphane Le Floch[3], Hélène Thomas-Guyon[1*]

Affiliations

[1]Littoral Environnement et Sociétés (LIENSs), UMR 6250, CNRS-Université de La Rochelle, 2 rue Olympe de Gouges – F-17042 La Rochelle Cedex 01, France. Email: thomas.milinkovitch01@univ-lr.fr ; helene.thomas@univ-lr.fr

[2]Institut National de l'Environnement Industriel et des Risques (INERIS), Unité d'écotoxicologie in vitro et in vivo, Parc Technologique ALATA – F-60550 Verneuil en Halatte, France. Email: Awa.Ndiaye@ineris.fr ; Wilfried.Sanchez@ineris.fr ;

[3]Centre de Documentation de Recherche et d'Expérimentations sur les Pollutions Accidentelles des Eaux (CEDRE), 715 rue Alain Colas, CS41836-F-29218 Brest Cedex 2, France. Email: stephane.le.floch@cedre.fr

*** Corresponding authors:** T. Milinkovitch; H. Thomas-Guyon

Littoral Environnement et Sociétés (LIENSs)

UMR 6250, CNRS-Université de La Rochelle

2 rue Olympe de Gouges

F-17042 La Rochelle Cedex 01, France

Email : thomas.milinkovitch01@univ-lr.fr

helene.thomas@univ-lr.fr

tel : +33 (0)5 46 50 76 48

fax : +33 (0)5 46 45 82 64

Abbreviations: BAL : Brut Arabian Light ; C : Control ; CD : Chemically Dispersed oil ; D : Dispersant solution ; MD : Mechanically Dispersed oil; WSF : Water Soluble Fraction

Abstract

Dispersant application is an oil spill response technique. To evaluate the environmental cost of this operation in nearshore habitats, the experimental approach conducted in this study exposed juvenile golden grey mullets (*Liza aurata*) for 48 hours to chemically dispersed oil (simulating, *in vivo*, dispersant application), to dispersant alone in sea water (as an internal control of chemically dispersed oil), to mechanically dispersed oil (simulating, *in vivo*, natural dispersion), to the water-soluble fraction of oil (simulating, *in vivo*, an oil slick confinement response technique) and to sea water alone (control condition). Biomarkers such as fluorescence of biliary polycyclic aromatic hydrocarbon (PAH) metabolites, total glutathione liver content, EROD (7-ethoxy-resorufin-O-deethylase) activity, liver antioxidant enzyme activity, liver lipid peroxidation and an innate immune parameter (haemolytic activity of the alternative complement pathway) were measured to assess the toxicity of dispersant application. Significant responses of PAH metabolites and total glutathione liver content to chemically dispersed oil were found, when compared to water-soluble fraction of oil. As it was suggested in other studies, these results highlight that priority must be given to oil slick confinement instead of dispersant application. However, since the same patterns of biomarkers responses were observed for both chemically and mechanically dispersed oil, the results also suggest that dispersant application is no more toxic than the natural dispersion occurring in nearshore areas (e.g. waves). The results of this study must, nevertheless, be interpreted cautiously since other components of nearshore habitats must be considered to establish a framework for dispersant use in nearshore areas.

Keywords: dispersed crude oil; dispersant; oxidative stress; complement system; *Liza aurata*; nearshore areas.

1. Introduction

By accelerating the dispersion of oil from the sea surface into the water column, the use of dispersants (surface active agents) offers the environmental benefits of (i) diluting the oil slick in the water column (Lessard and DeMarco, 2000), (ii) reducing the threat of oiling shorelines and (iii) accelerating the bacterial degradation of oil by increasing the available surface of the oil (Tiehm, 1994; Churchill et al., 1995). However, the use of dispersant is, at the moment, subject to certain restrictions depending mainly on weather conditions, oil type, distance to the shore and/or water depth. For example in European Atlantic coast the minimum permitted water depth is 10 m (Chapman et al., 2007). This restriction of minimum water depths was derived from studies on the dilution of dispersed oil in shallow water and took into consideration the ecological sensitivity of nearshore areas as they are nurseries for many aquatic species. However, a field study conducted by Baca et al. (2005) suggests that, in nearshore tropical ecosystems, dispersant use minimizes the environmental damages arising from an oil spill. This Net Environmental Benefits Analysis (NEBA) highlights a positive environmental role of dispersant use in nearshore areas but it is only applicable to tropical mangroves. To the best of our knowledge no NEBA has ever been conducted in Atlantic coastal ecosystems in order to establish the current restrictions for dispersant use and policies in nearshore areas. To do so, an on-going project (DISCOBIOL project: DISpersant and response techniques for COastal areas; BIOLogical assessment and contributions to the regulation) aims at obtaining informations on the environmental impact of dispersed oil in nearshore areas.

Including in this project, this study aims at assessing the toxicity of chemically dispersed oil at concentration similar to those encountered at oil spill sites. To simulate current operational oil dispersant application, our study uses a third generation dispersant, which is the more

recent formulations and is considered as the less toxic, the more concentrated in tensio-active and there by the most commonly used at the moment. While, most experimental studies assessed the toxicity of the dispersant itself (Adams et al., 1999; George-Ares and Clark, 2000) or the dispersed oil water-accommodated fraction (Cohen and Nugegoda, 2000; Mitchell and Holdway, 2000; Ramachandran et al., 2004; Perkins et al., 2005; Jung et al., 2009), our experimental approach simulates operational oil dispersant application, considering the presence of oil droplets in the water column. Indeed, oil droplets are suggested to be a determinant of toxicity (Brannon et al., 2006) and does so even more in nearshore areas, where natural dispersion (e.g. waves) can replace the whole oil slick from the surface in the water column (as described during the Braer oil spill by Lunel, 1995).

To reveal the toxicity of this chemically dispersed oil, several biomarkers were assessed after exposure of juvenile golden grey mullets (*Liza aurata)*. The choice of the species is due to (i) its presence in nearshore areas during its early life stages (Gautier and Hussenot, 2005) and consequently its status of pollutants target organism (Bruslé, 1981); and to (ii) its significant role in the coastal ecosystems, since this fish species permits an important particulate organic matter transport from the salt marsh to the marine coastal waters (Lafaille et al., 1998).

In this context, the use of biomarkers seems appropriate since they are defined as “a biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of one or more chemical pollutants” (Depledge et al., 1995). Hence, these ecotoxicological tools provide integrative informations, linking exposure to pollutants and the health of the monitored organisms (Sanchez and Porcher, 2009). As a consequence, other studies evaluate the toxicity to fish of a dispersed crude oil through biomarkers assessment (Cohen and Nugegoda, 2000; Jung et al., 2009; Mendonça Duarte et al., 2010) and reveal an increase of toxicity due to dispersant application. In our study, a set of complementary

biomarkers, including EROD (7-ethoxy-resorufin-O-deethylase) activity implicated in phase I biotransformation, total glutathione (GSH), enzymatic antioxidant activities (glutathione peroxidise, GPx; catalase, CAT; superoxide dismutase, SOD; glutathione-S-transferase, GST) and lipid peroxidation (LPO) were measured in the liver of golden grey mullet. These biomarkers are known to be sensitive to petroleum compounds and in particular to polycyclic aromatic hydrocarbons (PAHs) as described in Pan et al. (2005), Oliveira et al. (2008), Nahrgang et al. (2009) and Hannam et al. (2010). Moreover, the physiological links between the presence of PAHs, the production of radical oxygen species (ROS) and consequently enzymatic and non-enzymatic antioxidant responses have also been described (Stegeman, 1987; Livingstone, 2001). The haemolytic activity of the alternative complement pathway (ACH 50), an innate immune parameter that is involved in the innate humoral response, was measured in the plasma of the golden grey mullets, since it is a known biomarker of petroleum exposure (Bado-Nilles et al., 2009). Modulations of the antioxidant system and innate immune function will be discussed with regards to the 16 PAHs USEPA priority pollutants, the concentration of total petroleum hydrocarbons (TPHs) in seawater and exposure biomarkers: pyrene-derived and benzo[*a*]pyrene-derived biliary metabolites.

2. Materials and methods

2.1. Chemicals

An Arabian Crude Oil containing 54% saturated hydrocarbons, 36% aromatic hydrocarbons and 10% polar compounds, was selected for this study. Before exposure, the oil was evaporated (in a 1m^3 tank, during 24 hours) under atmospheric conditions and natural UV-sunlight in order to simulate the natural behaviour of the oil after it is released at sea

(evaporation of light compounds and natural photodegradation, respectively). The resulting chemical composition of the oil was 54% saturated hydrocarbons, 34% aromatic hydrocarbons and 12% polar compounds.

With regards to dispersant, a formulation manufactured by Total Fluides was selected based on its efficiency. Dispersant was evaluated by CEDRE (CEntre de Documentation de Recherche et d'Expérimentations sur les pollutions accidentelles des eaux, France) and was deemed effective enough to be used in the marine environment (preliminary determined using the method NF.T.90-345), non-toxic at the concentration recommended by the manufacturer (preliminary determined assessing standard toxicity test: method NF.T.90-349) and biodegradable. Its chemical formulation was not available for reasons of confidentiality.

2.2. Experimental animals

The experiment was carried out using 50 juvenile golden grey mullets (*Liza aurata*), which were provided by Commercio Pesca Novellame Srl, Chioggia, Italy. Their average length was 139.0 ± 0.7 mm (mean ± standard error of the mean) and their average weight was 38.25 ± 1.22 g.

The fish were acclimatized for 3 weeks in 300-L flow-through tanks (dissolved oxygen: 91 ± 2%; salinity: 35 ± 1%; 15 ± 0.1 °C, with a 12 h light:12 h dark photoperiod in seawater free of nitrate and nitrite) prior to the exposure studies. During acclimation, they were fed daily with fish food (Neosupra AL3 from Le Gouessant aquaculture) but were starved for 48 h prior to the bioassays and throughout the exposure period, in order to avoid bile evacuation from the gallbladder.

2.3. Experimental design

2.3.1. Experimental system

The experimental system (**Figure 1**) was devised to maintain the mixture of oil and dispersant as a homogenous solution. The mixture was homogenized using a funnel (at the surface of a 300-L seawater tank), which was linked to a Johnson L450 water pump (at the bottom of the tank) in order to homogenize the mixture despite the hydrophobic nature of the oil. Preliminary tests showed that, after 24 hours of homogenisation, the total petroleum hydrocarbon (TPH) concentrations in the water column do not depend on water column depth, suggesting the homogenous dispersion of small petroleum droplets throughout the water column. The system was a static water system stocked in a temperature controlled room (15 °C), and thus exposure studies were conducted at 15 ± 0.1 °C. Other physico-chemical parameters were also measured: pH (8.02 ± 0.07) and dissolved oxygen (95 ± 1%) remained constant throughout the study.

2.3.2. Exposure conditions and exposure media

Control exposure medium (C) was made up using seawater provided by Oceanopolis, Brest, France. The chemically dispersed (CD) oil exposure medium was made by pouring 20 g of petroleum and 1 g of dispersant into the funnel of the experimental system. Dispersant alone (D) exposure medium, as an internal control of CD, was made by pouring 1 g of dispersant into the funnel. The mechanically dispersed (MD) oil exposure medium was made by pouring 20 g of petroleum into this funnel. For the water-soluble fraction of oil (WSF), 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, a 20 g oil slick was contained using a plastic cylinder (21 cm diameter) placed on the surface of the seawater (4 cm below the surface and 8 cm above). A plastic mesh was placed at the bottom of the plastic cylinder. The spreading of the oil slick was not prevented by the plastic cylinder, as the oil slick was smaller in diameter than the plastic cylinder, therefore 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 and the fish were only exposed to the soluble fraction of the oil.

None of the fish were exposed for 24 hours, while the solutions remained homogenous. The groups of 5 fish were then randomly distributed in the five experimental tanks, each tank containing an exposure medium (described above). The fish were exposed to the different media for a period of 48 h and the protocol was replicated so that 10 fish were exposed to each exposure medium.

At the end of the exposure period, the fish in each tank (each exposure medium) were euthanized using eugenol (4-allyl-2-methoxyphenol). To collect plasma samples, 0.2 mL of blood was withdrawn from the caudal vein of each fish and centrifuged (12,000 g, 10 min, 4 °C, Jouan). Plasma samples were stored at –80 °C. The liver and gallbladder were removed from each fish and stored at –80 °C prior to analysis.

2.4. TPH and PAH concentrations

2.4.1. TPH seawater concentrations

The TPH concentration, which is the sum of dissolved hydrocarbon concentrations plus the amount of oil droplets, was measured for all exposure media at the beginning (T=0 h) and at

the end of fish exposure (T=48 h), using the mean of three replicated measurements for each time point. The seawater samples were extracted with 10 mL of pestipur-quality dichloromethane (99.8 % pure solvent, Carlo Erba Reactifs, SDS). After separation of the organic and aqueous phases, water was extracted two additional times with the same volume of dichloromethane (2 x 10 mL). The combined extracts were dried on anhydrous sulphate and then analyzed using a UV spectrophotometer (UV-Vis spectrophometer, Unicam) at 390 nm, as described by Fusey and Oudot (1976).

2.4.2. Seawater concentrations of PAHs

PAH concentrations were assessed at the beginning (T=0 h) and at the end of fish exposure (T=48 h), using the mean of three replicated measurements for each time point. After sampling, the first step was a 24-hour settling phase to separate oil droplets and particulate matter from the seawater. Then, PAHs were extracted from the seawater using the stir bar sorptive extraction technique (SBSE – Stir bar coated with PDMS, Gerstel), and analyzed using thermal desorption coupled to capillary gas chromatography-mass spectrometry (GC–MS). The GC was a HP7890 series II (Hewlett Packard, Palo Alto, CA, USA) coupled with a HP5979 mass selective detector (MSD, Electronic Impact: 70eV, voltage: 2 000 V). PAHs were quantified according to published procedures (Roy et al., 2005).

2.5. Biochemical analyses

2.5.1 Fixed wavelength fluorescence analysis

Bile samples were diluted (1:250) in absolute ethanol (VWR International). Fixed wavelength fluorescence (FF) was then measured at the excitation:emission wavelength pairs 341:383 and

380:430 nm. FF 341:383 mainly detects pyrene-derived metabolites and FF 380:430 mainly detects benzo[*a*]pyrene-derived metabolites (Aas et al., 2000). Measurements were performed in quartz cuvettes on a spectrofluorimeter (SAFAS Flx-Xenius). The FF values were expressed as arbitrary units of fluorescence and the signal levels of pure ethanol were subtracted.

2.5.2 Measurement of oxidative stress biomarkers

Livers were homogenized in ice-cold phosphate buffer (100 mM, pH 7.8) containing 20% glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a serine protease inhibitor. The homogenates were centrifuged at 10,000 g, 4 °C, for 15 min and the postmitochondrial fractions were used for biochemical assays. Total protein concentrations were determined using the method of (Bradford, 1976) with bovine serum albumin (Sigma-Aldrich Chemicals, France) as a standard. Hepatic biomarkers assays including GSH content and activities of EROD, GST, GPx, SOD and CAT were adapted for use in microplate and, after preliminary test using several dilutions, adapted for samples of liver of juvenile golden grey mullet.

The EROD activity was measured using the fluorimetric assay developed by Flammarion et al. (1998). To summarize, 10 µL of a 5g proteins/L diluted sample were added to phosphate buffer containing 8 µM of 7-ethoxyresorufin and 0.5 mM of NADPH. Formed resorufin was quantified by fluorimetric measurement with 530 nm wavelength excitation and 590 nm wavelength emission. Resorufin was used as standard, and results were expressed as nmol resorufin/min/g protein.

The GSH (total glutathione) concentration was measured according to Vandeputte et al. (1994). Briefly, 10 µL of TCA-deproteinized sample were mixed with phosphate buffer containing 0.3 mM NADPH and 1 mM Ellman reagent. The enzymatic reaction was

monitored spectrophotometrically at 405 nm and the results were expressed in µmol of GSH/g of proteins.

The GST activity assay was conducted according to Habig et al. (1974). Briefly, 10 µL of a 0.75 g proteins/L diluted sample were mixed with 1 mM chloro dinitro benzene and 1 mM reduced glutathione. The enzymatic reaction was monitored spectrophotometrically at 340 nm and the results were expressed in U of GST/g of proteins.

GPx activity was determined using 15 µL of a 4.5 g proteins/L diluted sample according to the method of Paglia and Valentine, (1967). Cumene hydroperoxide was used as the substrate and enzymatic activity was assessed at 340 nm. The results were expressed in U of GPx/g of proteins.

SOD activity was measured using the assay developed by Paoletti et al. (1986). Briefly, the inhibition of NADH (350 µM) oxidation by 20 µL of a 0.25 g proteins/L diluted sample was monitored at 340 nm. The results were presented in U of SOD/mg of proteins.

CAT activity was monitored using the method previously described by Babo and Vasseur (1992). Briefly, 0.08 g proteins/L diluted samples were mixed (v:v) with 28 mM hydrogen peroxide. The kinetics of hydrogen peroxide degradation were assessed at 280 nm and the results were expressed in U of CAT/mg of proteins.

2.5.3 Lipid peroxidation (LPO) determination

Lipid peroxidation levels were assessed via malondialdehyde (MDA) content determined using a commercially available MDA assay kit (Oxis International MDA assay kit). The method was based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA at 45 °C. The blue product was quantified by measuring absorbance at 586 nm (Gérard-Monnier et al., 1998).

2.5.4 Determination of the alternative pathway of plasma complement activity

Determination of the alternative pathway of plasma complement activity was carried out by haemolytic assay with rabbit red blood cells (RRC, Biomérieux, France) as described by Yano (1992) and adapted to microtitration plates. Plasma samples, diluted to 1/80 in EGTA-Mg-GVB buffer, were added in increasing amounts, from 10 to 100 µL per well. The wells were then filled with EGTA-Mg-GVB buffer to a final volume of 100 µL. Finally, 50 µL of a suspension containing 2% rabbit red blood cells were added to each well. Control values of 0% and 100% haemolysis were obtained using 100 µL of EGTA-Mg-GVB buffer and 100 µL of non-decomplemented trout haemolytic serum at 1/50 in ultra pure water respectively. Samples were incubated for 1 hour at 20 °C. The microplates were centrifuged (400 g, 5 min, 4 °C, Jouan). Then, 75 µL of supernatant from each well were transferred with 75 µL of phosphate buffer saline (Biomérieux, France) into another 96-well microplate. The absorbance (540 nm) was read in a spectrofluorimeter (SAFAS Flx-Xenius) and the number of ACH 50 units per mL of plasma was determined by reference to 50% haemolysis.

2.6 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. Firstly, Student's t-tests were conducted, for each variables (fixed wavelength fluorescence, EROD activity, total glutathione concentration, hepatic oxidative stress biomarkers, lipid peroxidation, haemolytic activity of alternative complement pathway) in order to highlight significant differences between both experimental replicates of each

exposure media. No significant difference was found, thereby, both replicates were considered as one homogenous group of ten individuals. A factorial analysis of variance (one-way ANOVA) was performed in order to assess the effects of the several exposure conditions. This statistical analysis was followed by the Tukey post-hoc test to detect significant differences between groups. The significance of the results was ascertained at $\alpha=0.05$. The results were expressed as means ± s.e.m. (standard error of the mean) corresponding to groups of ten fish (n=10).

3. Results

No fish mortality was observed during the experiments. Moreover no TPH or PAH was detected in the control and dispersant exposure media. The TPH concentration measured in the CD (chemically dispersed oil) and MD (mechanically dispersed oil) groups corresponded to that encountered under oil spill situations (for instance, 1 to 100 mg/L of total petroleum hydrocarbons were measured in coastal waters around Shetland during the Braer oil spill, as reported by Lunel,1995). No oil slick was observed in either the CD or MD exposure media, suggesting that the energy in the experimental system was sufficient to disperse the oil slick. These observations validated the experimental procedure.

3.1. Total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAH) concentration in seawater.

The TPH concentrations of oil were higher in media with dispersant compared to without, and the lowest concentration was observed in the WSF (water soluble fraction of oil) medium, in

which only dissolved compounds were present in the seawater column. In the CD medium, the TPH concentration (**Table 1**) was 39 mg/L at the beginning of the exposure period (T=0 h) and 25 mg/L at the end of the exposure period (T=48 h), giving a percentage decrease of 36 %. In the MD medium, the TPH concentration was 13 mg/L at the beginning of the exposure period (T=0 h) and 9 mg/L at the end of the exposure period (T=48 h), giving a percentage decrease of 29 %. The TPH concentration could not be determined in the WSF medium since it was too low to be detected using spectrophotometry.

According to spectrophotometry as well as gas chromatography coupled with mass spectrometry, petroleum compounds and PAHs were not detected in the D (Dispersant) or C (Control) media.

In terms of the sum of 16 parent and alkylated USEPA PAHs (ΣPAH) concentrations, the CD medium contained 43.98 μg/L, at the beginning of the experiment, then 26.34 μg/L after 48 hours, giving a percentage decrease of 40 %. For MD, the percentage decrease was 48 %: the ΣPAH concentration at T=0 h was 39.09 μg/L and at T=48 h it was 20.63 μg/L. WSF values were lower when compared to both the CD and MD values, with a ΣPAHs concentration at T=0 h of 5.16 μg/L and at T=48 h of 0.47 μg/L, corresponding to a drastic decrease (91 %).

Regarding the concentration of 16 USEPA PAHs (alkylated and parents) in seawater during CD, MD and WSF exposures (**Table 2**), it appears that two- or three-ring PAH compounds (specifically, naphthalene alkylated compounds) were dominant when compared to heavier PAHs (≥ four rings). Regarding the variation over time in PAH concentration, it appears that light PAHs such as naphthalene (parent and alkylated) decreased during CD, MD and WSF exposure (with the exception of fluorene for CD exposure) while the concentrations of heavier PAHs remained relatively stable or increased (e.g. chrysene).

3.2. Fixed wavelength fluorescence analysis of biliary PAH metabolites

With regards to the levels of benzo[*a*]pyrene-type metabolites, which were measured by fluorescence intensity (FF 380:430), CD and MD exposures led to significantly higher values, compared to the values obtained in control fish (C). The intensity of fluorescence did not significantly differ between the C, WSF and D groups of fish, even though WSF exposure seemed to increase the intensity (**Figure 2a**).

With regards to the levels of pyrene-type metabolites (**Figure 2b**), which were measured by fluorescence intensity (FF 341:383), CD and MD exposure led to significantly higher values when compared to values obtained in control fish (C). The intensity of fluorescence did not significantly differ between the C, WSF and D groups of fish, even though WSF exposure seemed to increase the intensity. The intensity of fluorescence following CD exposure was significantly different to that following WSF and D exposure while it appears that MD exposure did not induce an increase in fluorescence compared to WSF and D exposure.

3.3. EROD (7-ethoxy-resorufin-O-deethylase) activity, Total glutathione content and hepatic oxidative stress biomarkers

EROD demonstrated no significant difference between the exposure conditions **(Figure 3)** and was characterized by a high intragroup variability that could reflect differences in biotransformation processes between organisms.

The concentration of GSH (total glutathione, **Figure 4**) significantly decreased after exposure to CD (45.35 ± 8.65) and MD (53.18 ± 10.04), compared to the control group (130.50 ± 32.64), while no significant difference was observed after exposure to WSF (90.51 ± 23.11) or D (108.44 ± 22.86). When CD and MD were compared with WSF and D, no significant

difference was revealed even though the GSH content in the WSF and D groups seemed higher than in the CD and MD groups.

No significant difference between exposure conditions was recorded for antioxidant enzyme activities (i.e. GST, GPx, SOD and CAT) (**Figure 5**).

3.4. Lipid peroxidation (LPO)

As for antioxidant enzymes, LPO demonstrated no significant difference between the exposure conditions **(Figure 6)**. However, LPO was characterized by a high intragroup variability (especially for WSF, CD and MD exposure media) that could reflect differences in sensitivity between organisms.

3.5. Haemolytic activity of alternative complement pathway (ACH 50)

The results are presented in **Figure 7**. As for antioxidant enzymes, ACH 50 demonstrated no significant difference between the exposure conditions. Haemolytic activity appeared to be lower after CD exposure and higher after MD exposure.

4. Discussion

The aim of this study was to accurately simulate operational oil dispersant application and to assess its toxicity. An experimental system providing mixing energy (described in section 2.3.1) was necessary for this purpose: to achieve the dispersion of crude oil through operational dispersant application, seawater energy is necessary (Merlin, 2005). Readers must take into account that the results obtained (and discussed below), through this experimental

approach, are available only for a given mixing energy (the mixing energy induces by the waterpump). However, extrapolation of results from the experimental approach to the oil spill operations is possible. Indeed, meteorological conditions during the Braer oil spill (Wind force 7 to 10, Lunel, 1995) were the most propitious to dispersed oil, among most of the meteorological conditions during oil spills. While a dispersion of the whole oil was maintained for more than one week, other oil spills, in offshore areas, exposed an unstable dispersion of oil slick with a rapid decrease of concentration in 2-5 hours (Lessard and Demarco, 2000). Our experimental approach is situated between these two opposite scenarios (decrease of concentration on a 48 hours period, discussed in 4.1) and thus can be considered as a possible one. Moreover, acccording to CEDRE observations during oil spill response in nearshore area, 4 tide cycles (48h) are sufficient to totally disperse the oil slick, so that no petroleum is present after this period. This suggests that an exposure of 48 h seems to be accurate.

The fish were exposed to (i) a chemically dispersed oil (simulating dispersant application), (ii) dispersant alone in sea water (as an internal control of chemically dispersed oil), (iii) mechanically dispersed oil (simulating natural dispersion), (iv) water-soluble fraction of oil (simulating an oil slick confinement response technique) and to (v) sea water alone (control condition).

.

4.1. Total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAH) concentrations in seawater.

The energy supplied by the experimental system was the same for the five exposure media. However, our results show that the TPH concentration in the water column was higher in CD than in MD at T=0 h and T=48 h. This finding and our observations, suggest that oil adheres

more to the experimental system in the MD exposure medium than in the CD exposure medium. When extrapolated to field operations in the shallow water of nearshore areas, the results show that the application of dispersants would promote higher concentrations of TPH in the water column but would decrease the adherence to substrates (seagrass beds, sediments etc…). This result is in accordance with Baca et al. (2005) and shows that dispersant application increases the exposure to TPH for pelagic organisms living in the water column (as golden grey mullets), while decreases the exposure to TPH for benthic organisms.

Unlikely TPH concentration, the difference of the sum of PAH concentrations between CD and MD exposures is low (slightly higher in CD exposure). The sum of PAH concentration is relevantly lower in WSF exposure medium than in CD and MD exposures (at T=0 h and T=48 h): as a consequence of dispersion, oil droplets have a larger surface-to-volume ratio than an oil slick, and this would accelerate the solubilization of PAHs in seawater. Consideration must also be given to the fact that the sum of PAHs decreased slightly in the CD and MD exposure media while drastically decreased during the 48 hours of WSF exposure media. The solubilization and volatilization/photolysis of PAHs are two opposing processes that determine the distribution and the residence time of PAHs in seawater (Schwarzenbach et al., 2003). In this case, it can be hypothesized that the dispersion of oil (CD and MD exposure) triggers the solubilization of PAHs from oil droplets into the seawater, which relatively compensates for the volatilization/photolysis of PAHs that occurs during the exposure. Inversely the solubilization of PAHs from the oil slick to the seawater (WSF exposure) was not high enough to compensate for the loss of PAHs due to volatilization/photolysis. Another explanation could be that PAH loss is due to absorption by golden grey mullets as it is suggested in literature for other organisms (Le Floch et al. 2003; Goanvec et al. 2008).

With regards to the 16 USEPA PAHs (alkylated and parent), the results show that light PAHs (two to three rings) were predominant in the WSF, CD and MD exposure media at T=0 h and

T=48 h. This observation is consistent with the current theory that the aqueous solubility increases as the molecular weight of PAHs decreases (Neff, 1979). Moreover, with the exception of fluorene, the concentrations of light PAHs decreased during the experiment while the concentration of heavy PAHs remained stable (cf. Indeno[*1,2,3-cd*]pyrene and Dibenzo[*a,h*]anthracene in **Table 2**), a phenomenon probably attributable to the volatilization/photolysis of light PAHs (Schroeder and Lane, 1988).

4.2. Fixed wavelength fluorescence analysis of biliary PAH metabolites

The fixed wavelenght fluorescence of fish biliary metabolites has been used as a PAHs exposure biomarker in many studies (Aas et al., 2000; Barra et al., 2001; Kopecka-Pilarczyk and Correia, 2009; Insausti et al., 2009).

In our study pyrene-derived fluorescence was significantly higher under MD and CD exposures than under control exposure (C). However, only fluorescence under CD exposure was significantly higher than WSF and D exposures, which show that the exposure to pyrene was higher when the oil was chemically dispersed. These results are consistent with the alkylated fluoranthenes/pyrenes seawater concentration at T=48 h since this was higher under CD exposure. However at T=0 h, no pyrene (alkylated or parent) was detected under CD. Concerning the benzo[*a*]pyrene-type metabolites, fluorescence was higher under CD and MD exposures than for the other exposure groups (WSF, D, C), indicating a higher bioavailability of this PAH (and its derived type). Even though the relative fluorescence was higher under WSF exposure than in other conditions (D and C), the difference was not significant. These results are consistent with the benzo[*a*]pyrene concentrations measured in the seawater, since the concentration of this PAH was similar for CD and MD exposures and lower for WSF exposure (at T=0 h and T=48 h). Benzo[*a*]pyrene is considered carcinogenic and is a radical

oxygen species producer through its role as a P450 mixed-function oxidase (MFO) inducer (Lemaire-Gony and Lemaire, 1993). This result is of importance because it reveals the potentially high toxicity of CD and MD exposures when compared to other conditions.

For both metabolite types, the relative fluorescence revealed a higher exposure of fish to PAHs under CD exposure (compared to WSF), probably resulting from the higher PAH concentrations in the seawater. The results are consistent with the literature; indeed Ramachandran et al. (2004) showed that oil dispersant increases PAHs uptake by fish exposed to crude oil. Moreover Jung et al. (2009) showed that hydrocarbons metabolites in bile from fish exposed to crude oil treated with dispersant were significantly higher compared with fish exposed to crude oil alone.

To the best of our knowledge no studies have been conducted in order to allow the comparison between the toxicity of an oil slick dispersed with turbulent mixing energy and dispersant (CD) to an oil slick dispersed only with turbulent mixing energy (MD). Even if benzo[*a*]pyrene derived metabolites levels seem to be slightly lower in MD exposure than in CD exposure, no significant difference was highlighted. This is in accordance with the benzo[*a*]pyrene concentrations in seawater (no difference between CD and MD exposure). However, for pyrene derived metabolites, while a significant difference was observed between CD and WSF, no difference was observed between MD and WSF exposure. This finding is in accordance with the pyrene concentration in sea water: alkylated fluoranthenes/pyrenes seawater concentration (at T=48 h) was higher under CD exposure.

4.3. EROD (7-ethoxy-resorufin-O-deethylase) activity

Since the eighties, EROD activity is commonly used to reveal PAHs biotransformation (Addison and Payne, 1987) and thereby a large body of literature permits comparison of our

results to other studies. Furthermore, since EROD activity is involved in phase I biotransformation of xenobiotics, the modulation of this biomarker in response to PAHs is more precociously observed than the increase of PAHs biliary metabolites (described above). By the way EROD activity measurement gives an idea of organism short term defence against the xenobiotics.

Ramachandran et al. (2004) and Jung et al. (2009) showed an increase of EROD activity following chemically dispersed oil exposure. However, our study did not show an EROD activity increase while a PAHs biliary metabolites increase was observed following dispersed crude oil exposure. A reason for this lack of significance could be the low sensitivity to PAHs of EROD activity, when compared to biliary metabolites sensitivity (Camus et al., 1998).

4.4. Total glutathione content

The results obtained for total glutathione content in the liver of *Liza aurata* after 48 h confirmed previous results concerning biliary metabolites contents since a significant difference was found between dispersed oil exposure (CD and MD) and the control condition. These results are consistent with the literature since Almroth et al. (2008) showed a significance decrease in total glutathione in corkwing wrasse (*Symphodus melops*) exposed to contaminated PAHs sites. The total glutathione content, which corresponds to reduced plus oxidized glutathione (GSH+GSSG), was lower in both conditions (CD and MD), although GST activity did not change. This finding shows that depletion was not due to glutathione conjugation (phase II detoxification) since an increase in GST should be concomitant with conjugation. Nevertheless, it is possible that the decrease in total glutathione was due to inhibition of the GSH synthesis rate by contaminants, as suggested in Canesi et al. (1999), in Wang et al. (2008) and in Zhang et al. (2004) on freshwater crabs, mussels and goldfish,

respectively. Another explanation could be that, in the process of detoxification, reduced glutathione chelated the heavy metals contained in petroleum (mainly vanadium and nickel) so that GS-V or GS-Ni binding complexes are formed (Sies, 1999). These complexes cannot be assessed through biochemical analysis and contributed to the observed reduction in total glutathione content. However, according to low heavy metals concentration in common crude oil (e.g. 109.9 mg/L of Vanadium and 71.5 mg/L of Nickel, Salra Amoli et al., 2006) and the short exposure period of our experiment, this explanation seems to be less accurate.

So, although the mechanism is not fully understood, this study shows that total glutathione is depleted, suggesting, for CD and MD exposures, a reduction in the first line cellular defence, since glutathione is involved in several detoxification reactions. Indeed, conjugation of glutathione to contaminants can prevent them from interacting deleteriously with other cellular components, enabling the organism to cope with the contaminated environment (Maracine and Segner, 1998).

Moreover Ringwood and Conners (2000) showed that gonadal depletion of glutathione induces a decrease in reproductive success in oyster. Even if this study was conducted in oyster, this finding suggests that a link between the total glutathione pool and the organism fitness could exist. Since our study demonstrated a depletion of the total glutathione pool in the liver of juveniles golden grey mullets, it would also be interesting to assess the total glutathione in the gonads of adult fish.

4.5 Antioxidant enzyme activity and lipid peroxidation (LPO).

Antioxidant enzyme activity has been shown to be modulated in response to short term (≤48 h) contaminants exposure in different targets organs of fish (Ahmad et al., 2005; Sun et al., 2006; Modesto and Martinez, 2010) and especially to short term PAHs exposure in the liver

of golden grey mullet (Oliveira et al., 2008). However, in our study, results concerning antioxidant enzyme activity showed no significant differences between exposure conditions, suggesting that oxidative stress was absent.

LPO was measured via the malondialdehyde content in the liver and revealed the targeting of cell membranes by radical oxygen species (ROS), thus altering membrane fluidity, compromising membrane integrity, inactivating membrane-bound enzymes and disrupting surface receptor molecules. In Ahmad et al. (2005) and in Oliveira et al (2008), a LPO increase was observed in fish gills after 48 h of contamination and in fish livers after 16 h of contamination, respectively. In our study the high intragroup variability, when compared to other studies (Oliveira et al., 2008; Kopecka-Pilarczyk and Correia, 2009), induced a lack of significance, confirming the notion that oxidative stress was absent. However it should be stated that, for exposure conditions containing petroleum (CD, MD, WSF) a high intragroup variability was observed whereas a lower variability was observed following Control (C) and single dispersant (D) exposure. This observation suggests a difference of oxidative stress between the individuals exposed to conditions containing petroleum.

Oliveira et al. (2008) evaluated oxidative stress using, as in our study, LPO and antioxidant enzyme activity in the liver of *Liza aurata* exposed to a PAH (phenanthrene). They found a significant difference in these biomarkers, but the concentrations of phenanthrene were 50 to 1300 times higher than in our study.

4.6 Haemolytic activity of alternative complement pathway (ACH 50)

The innate immune function has also been used as a biomarker of PAH toxicity (Seeley and Weeks-Perkins, 1997; Carlson et al., 2004). The complement system of teleost fish is a powerful defence system since it is involved in important immune functions that are pivotal to

the recognition and clearance of microbes (Boshra et al., 2006). Moreover, the haemolytic activity of the alternative complement pathway has been shown to be a suitable biomarker of PAHs contamination in teleost fish (Bado-Nilles et al., 2009). On this basis, the alternative complement pathway was chosen since its functional degradation by exposure to petroleum compounds could reveal an alteration in fish health.

In our study no significant difference was found between the control condition and contaminant exposures, even though the haemolytic activity seemed to be lower following CD exposure. Bado-Nilles et al. (2009) found significant differences between contaminated and control fish, for a sum of PAH concentrations that was lower than in our study, but for longer exposure times, suggesting that alteration of haemolytic activity could have been observed after more than 48 h of exposure.

5. Conclusion

Based on fixed wavelength fluorescence analysis of biliary PAH metabolites, the results from this study show higher exposure for dispersed crude oil (CD and MD) than for other types of contaminant exposure. Also, the total glutathione content, described as a first line cellular defence against contaminants, was significantly reduced under dispersed oil exposures. Antioxidant enzymes did not show any responses to the contamination. EROD activity, lipid peroxidation and the haemolytic activity of the complement system also did not respond when fish were exposed to contaminants.

These results demonstrate a significant response of biomarkers to chemically dispersed oil, when compared to a non-dispersed oil slick (water-soluble fraction of oil), suggesting that oil slicks must not be dispersed when containment and recovery can be conducted at the oil spill site (low mixing energy of seawater). This finding is in accordance with an important body of

literature: many studies show an increase of PAHs toxicity to fish following dispersant application (Perkins et al., 1973; Cohen and Nugegoda, 2000; Ramachandran et al., 2004; Lin et al., 2009). On the other hand, no significant difference in the response of biomarkers was observed between chemically and mechanically dispersed oil. This finding suggests that when containment and recovery cannot be conducted (high mixing energy of seawater) the application of dispersant in nearshore areas is no more toxic than the natural dispersion (wave, current, swell).

To conclude, the results of this study are of interest with regards establishing a framework for dispersant use and policies in nearshore areas since they are part of a current project: DISCOBIOL project (DISpersant and response techniques for COastal areas: BIOLogical assessement and contributions to the regulation). Initially, this project intends to assess the toxicity of chemically dispersed oil to several species living in nearshore areas (*Crassostera gigas*, *Mytilus edulis*, *Scophtalmus maximus*, *Dicentrarchus labrax* and *Liza aurata*). For this reason, organisms were exposed to oil in the water column. However, since dispersed crude oil can interact with other components of nearshore area habitats, such as mudflats, further studies must be conducted in order to better evaluate the net environmental benefits of dispersant application in nearshore areas.

Acknowledgements

This study was supported by a PhD grant from the Conseil Général of the Charente-Maritime. The Agence Nationale de la Recherche and especially Michel Girin and Gilbert Le Lann are acknowledged for financial support for the project ‘DISCOBIOL’, managed by F. X. Merlin. The authors also acknowledge Total Fluides and Innospech for providing chemicals. Special

thanks go to Julie Lucas and Marion Menguy for their help and assistance during the study and during the experimental procedures.

References

Aas, E., Baussant, T., Balk, L., Liewenborg, B., Andersen, O.K., 2000. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod. Aquat. Toxicol. 51, 241-258.

Adams, G.G., Klerks, P.L., Belanger, S.E., Dantin, D., 1999. The effect of the oil dispersant omni-clean on the toxicity of fuel oil n° 2 in two bioassays with the sheepshead minnow cyprinodon variegatus. Chemosphere 39, 2141-2157.

Addison, R.F., Payne, J.F., 1987. Assessment of hepatic mixed function oxidase induction in winter flounder (*Pseudopleuronectes americanus*) as a marine petroleum pollution monitoring technique, with an appendix describing practical field measurements of MFO activity. Can. Tech. Rep. Fish Aquat. Sci. n°150.

Almroth, B.C., Sturve, J., Stephensen, E., Holth, T.F., Förlin, L., 2008. Protein carbonyls and antioxidant defenses in corkwing wrasse (Symphodus melops) from a heavy metal polluted and a PAH polluted site. Mar. Environ. Res. 66, 271-277.

Babo, S., Vasseur, P., 1992. *In vitro* effects of Thiram on liver antioxidant enzyme activities of rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 22, 61-68.

Baca, B., Ward, G.A., Lane, C.H., Schuler, P.A., 2005. Net environmental benefit analysis (NEBA) of dispersed oil on nearshore tropical ecosystems derived from International oil spill conference, Miami, Florida, USA, pp. 1-4.

Bado-Nilles, A., Quentel, C., Auffret, M., Le Floch, S., Gagnaire, B., Renault, T., Thomas-Guyon, H., 2009. Immune effects of HFO on European sea bass, *Dicentrarchus labrax*, and Pacific oyster, *Crassostrea gigas*. Ecotoxicol. Environ. Saf. 72, 1446-1454.

Barra, R., Sanchez-Hernandez, J.C., Orrego, R., Parra, O., Gavilan, J.F., 2001. Bioavailability of PAHs in the Biobio river (Chile): MFO activity and biliary fluorescence in juvenile *Oncorhynchus mykiss*. Chemosphere 45, 439-444.

Boshra, H., Li, J., Sunyer, J.O., 2006. Recent advances on the complement system of teleost fish. Fish Shellfish Immunol. 20, 239-262.

Bradford, M.M., 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Brannon, E.L., Collins, K.M., Brown, J.S., Neff, J.M., Parker, K.R., Stubblefield, W.A., 2006. Toxicity of weathered "Exxon Valdez" crude oil to pink salmon embryos. Environ. Toxicol. and Chem. 25, 962-972.

Bruslé, J., 1981. Food and feeding in grey mullets. in: Oren O.H. (ed.), Aquaculture of grey mullets. Cambridge University Press, Cambridge, pp. 185-217.

Camus, L., Aas, E., Børseth, J.F., 1998. Ethoxyresorufin-O-deethylase activity and fixed wavelength fluorescence detection of PAHs metabolites in bile in turbot (*Scophthalmus maximus L.*) Exposed to a dispersed topped crude oil in a Continuous Flow System. Mar. Environ. Res. 46, 29-32.

Canesi, L., Viarengo, A., Leonzio, C., Filippelli, M., Gallo, G., 1999. Heavy metals and glutathione metabolism in mussel tissues. Aquat. Toxicol. 46, 67-76.

Carlson, E.A., Li, Y., Zelikoff, J.T., 2004. Suppressive effects of benzo[a]pyrene upon fish immune function: Evolutionarily conserved cellular mechanisms of immunotoxicity. Mar. Environ. Res. 58, 731-734.

Chapman, H., Purnell, K., Law, R.J., Kirby, M.F., 2007. The use of chemical dispersants to combat oil spills at sea: A review of practice and research needs in Europe. Mar. Pollut. Bull. 54, 827-838.

Churchill, P.F., Dudley, R.J., Churchill, S.A., 1995. Surfactant-enhanced bioremediation. Waste Manag. 15, 371-377.

Cohen, A. M., Nugegoda, D., 2000. Toxicity of Three Oil Spill Remediation Techniques to the Australian Bass (*Macquaria novemaculeata*). Ecotoxicol. Environ. Saf. 47, 178-185.

Depledge, M.H., Aagaard, A., Györkös, P., 1995. Assessment of trace metal toxicity using molecular, physiological and behavioural biomarkers. Mar. Pollut. Bull. 31, 19-27.

Flammarion, P., Migeon, B., Garric, J., 1998. Statistical analysis of cyprinid ethoxyresorufin-O-deethylase data in a large French watershed. Ecotoxicol. Environ. Saf. 40, 144-153.

Fusey, P. and Oudot, J. 1976. Comparaison de deux méthodes d'évaluation de la biodégradation des hydrocarbures in vitro. *Mater. U. Organ.*, 4, p.241-251.

Gautier, D., Hussenot, J. (Eds.), 2005. Les mulets des mers d'Europe. Synthèse des connaissances sur les bases biologiques et les techniques d'aquaculture. Ifremer, Paris.

George-Ares, A., Clark, J.R., 2000. Aquatic toxicity of two Corexit dispersants. Chemosphere 40, 897-906.

Gérard-Monnier, D., Erdelmeier, I., Régnard, K., Moze-Henry, N., Yadan, J., Chaudière, J., 1998. Reactions of 1-Methyl-2-phenylindole with Malondialdehyde and 4-Hydroxyalkenals. Analytical Applications to a Colorimetric Assay of Lipid Peroxidation. Chem. Res. Toxicol. 11, 1176-1183.

Goanvec, C., Theron, M., Lacoue-Labarthe, T., Poirier, E., Guyomarch, J., Floch, S.L., Laroche, J., Nonnotte, L., Nonnotte, G., 2008. Flow cytometry for the evaluation of chromosomal damage in turbot *Psetta maximus* (L.) exposed to the dissolved fraction of heavy fuel oil in sea water: a comparison with classical biomarkers. J. Fish. Biol. 73, 395-413.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-Transferases. The first enzymatic step in mercapturic acid formation. J. of Biol. Chem. 249, 7130-7139[2].

Hannam, M.L., Bamber, S.D., Galloway, T.S., John Moody, A., Jones, M.B., 2010. Effects of the model PAH phenanthrene on immune function and oxidative stress in the haemolymph of the temperate scallop *Pecten maximus*. Chemosphere 78, 779-784.

Insausti, D., Carrasson, M., Maynou, F., Cartes, J.E., Solé, M., 2009. Biliary fluorescent aromatic compounds (FACs) measured by fixed wavelength fluorescence (FF) in several marine fish species from the NW Mediterranean. Mar. Pollut. Bull. 58, 1635-1642.

Jung, J.H., Yim, U.H., Han, G.M., Shim, W.J., 2009. Biochemical changes in rockfish, Sebastes schlegeli, exposed to dispersed crude oil. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 150, 218-223.

Kopecka-Pilarczyk, J., Correia, A.D., 2009. Biochemical response in gilthead seabream (Sparus aurata) to *in vivo* exposure to a mix of selected PAHs. Ecotoxicol. Environ. Saf. 72, 1296-1302.

Laffaille, P., Brosse, S., Feunteun, E., Baisez, A., Lefeuvre, J.C., 1998. Role of fish communities in particulate organic matter fluxes between salt marshes and coastal marine waters in the Mont Saint-Michel Bay. Hydrobiologia 373/374**,** 121–133.

Le Floch, S., Guyomarch, J., Merlin, F., Borseth, J., Corre, P.L., Lee, K., 2003. Effects of oil and bioremediation on mussel (*Mytilus edulis* L.) growth in mudflats. Environ. Technol. 24, 1211-1219.

Lemaire-Gony, S., Lemaire, P., 1993. Interactive effects of cadmium and benzo(a)pyrene on cellular structure and biotransformation enzymes of the liver of the European eel *Anguilla anguilla*. Aquat. Toxicol. 22, 145-160.

Lessard, R.R., DeMarco, G., 2000. The Significance of Oil Spill Dispersants. Spill Sci. Technol. Bull. 6, 59-68.

Lin, C.Y., Anderson, B.S., Phillips, B.M., Peng, A.C., Clark, S., Voorhees, J., 2009. Characterization of the metabolic actions of crude versus dispersed oil in salmon smolts via NMR-based metabolomics. Aquatic. Toxicol. 95, 230-238.

Livingstone, D.R., 2001. Contaminant-stimulated Reactive Oxygen Species Production and Oxidative Damage in Aquatic Organisms. Mar. Pollut. Bull. 42, 656-666.

Lunel, T., 1995. The Braer oil spill: oil fate governed by dispersion International oil spill conference, Long Beach, California, USA

Maracine, M., Segner, H., 1998. Cytotoxicity of metals in isolated fish cells: Importance of the cellular glutathione status. Comparative Biochemistry and Physiology Part A 120, 83–88.

Mendonça Duarte, R., Tomio Honda, R., Luis Val, A., 2010. Acute effects of chemically dispersed crude oil on gill ion regulation, plasma ion levels and haematological parameters in tambaqui (*Colossoma macropomum*). Aquat. Toxicol. 97, 134–141.

Merlin, F.X., 2005. Traitement aux dispersants des nappes de pétrole en mer. CEDRE (CEntre de Documentation de Recherche et d'expérimentations sur les pollutions accidentelles des eaux), Brest, France.

Mitchell, F.M., Holdway, D.A., 2000. The acute and chronic toxicity of the dispersants Corexit 9527 and 9500, water accommodated fraction (WAF) of crude oil, and dispersant enhanced WAF (DEWAF) to *Hydra viridissima* (green hydra). Water Res. 34, 343-348.

Modesto K.A., Martinez C.B.R., 2010. Roundup causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus lineatus*. Chemosphere 78, 294–299.

Nahrgang, J., Camus, L., Gonzalez, P., Goksøyr, A., Christiansen, J.S., Hop, H., 2009. PAH biomarker responses in polar cod (*Boreogadus saida*) exposed to benzo(a)pyrene. Aquat. Toxicol. 94, 309-319.

Neff, J.M. (Ed.), 1979. Polycyclic Aromatic Hydrocarbons in the Aquatic Environment: Sources, Fates and Biological Effects. Applied Science Publishers Ltd., Essex, England.

Oliveira, M., Pacheco, M., Santos, M.A., 2008. Organ specific antioxidant responses in golden grey mullet (*Liza aurata*) following a short-term exposure to phenanthrene. Sci. Total Environ. 396, 70-78.

Paglia, D.E., Valentine, W.N., 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70, 158-169.

Pan, L., Ren, J., Liu, J., 2005. Effects of benzo(k)fluoranthene exposure on the biomarkers of scallop Chlamys farreri. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 141, 248-256.

Paoletti, F., Aldinucci, D., Mocali, A., Caparrini, A., 1986. A sensitive spectrophotometric method for the determination of superoxide dismutase activity in tissue extracts. Anal Biochem. 154, 536-541.

Perkins, R.A., Rhoton, S., Behr-Andres, C., 2005. Comparative marine toxicity testing: A cold-water species and standard warm-water test species exposed to crude oil and dispersant. Cold Regions Sc.Technol. 42, 226-236.

Ramachandran, S.D., Hodson, P.V., Khan, C.W., Lee, K., 2004. Oil dispersant increases PAH uptake by fish exposed to crude oil. Ecotoxicol. Environ. Saf. 59, 300-308.

Ringwood, A.H., Conners, D.E., 2000. The effects of glutathione depletion on reproductive success in oysters, *Crassostrea virginica*. Mar. Environ. Res. 50, 207-211.

Roy, G., Vuillemin, R., Guyomarch, J., 2005. On-site determination of polynuclear aromatic hydrocarbons in seawater by stir bar sorptive extraction (SBSE) and thermal desorption GC-MS. Talanta 66, 540-546.

Salar Amoli, H., Porgamb, A., Bashiri Sadr, Z., Mohanazadeh, F., 2006. Analysis of metal ions in crude oil by reversed-phase high performance liquid chromatography using short column. J. Chromato. 1118, 82–84.

Sanchez, W., Porcher, J.-M., 2009. Fish biomarkers for environmental monitoring within the Water Framework Directive of the European Union. TrAC, Trends Anal. Chem. 28, 150-158.

Schroeder, W.H., Lane, D.A., 1988. The fate of toxic airborne pollutants. Environ. Sci Technol. 22, 240-246

Schwarzenbach, R.P., Gschwend, P.M., Imboden, D.M. (Eds.), 2003. Environmental Organic Chemistry (second ed.). Wiley InterScience, Hoboken, USA.

Seeley, K.R., Weeks-Perkins, B.A., 1997. Suppression of natural cytotoxic cell and macrophage phagocytic function in oyster toadfish exposed to 7,12-dimethylbenz[a]anthracene. Fish Shellfish Immunol. 7, 115-121.

Sies, H., 1999. Glutathione and its role in cellular functions. Free Radical Biol. Med. 27, 916-921.

Stegeman, J.J., 1987. Cytochrome P450 isozymes and monoxygenase activity in aquatic animals. Environ. Health Perspect. 71, 87-95.

Sun, Y., Yu, H., Zhang, J., Yin, Y., Shi H., Wang, X., 2006. Bioaccumulation, depuration and oxidative stress in fish (*Carassius auratus*) under phenanthrene exposure. Chemosphere 63, 1319–1327

Tiehm, A., 1994. Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. Appl. Environ. Microbiol. 60, 258-263.

Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57-149.

Vandeputte, C., Guizon, I., Genestie-Denis, I., Vannier, B., Lorenzon, G., 1994. A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. Cell Biol. Toxicol. 10, 415-421.

Wang, L., Yan, B., Liu, N., Li, Y., Wang, Q., 2008. Effects of cadmium on glutathione synthesis in hepatopancreas of freshwater crab, *Sinopotamon yangtsekiense.* Chemosphere 74, 51–56

Xue, W., Warshawsky, D., 2005. Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: A review. Toxicol. Appl. Pharmacol. 206, 73-93.

Yano, T., 1992. Assays of hemolytic complement activity. in: Stolen, J.S., Fletcher, T.C., Anderson, D.P., Kaattari, S.L., Rowley, A.F. (Eds.). Techniques in Fish Immunology. SOS Publications, Pair Haven, New Jersey, USA, pp. 131-142.

Yin, Y., Jia, H., Sun, Y., Yu, H., Wang, X., Wu, J., Xue, Y., 2007. Bioaccumulation and ROS generation in liver of *Carassius auratus*, exposed to phenanthrene. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 145, 288-293.

Zhang, J.F., Wang, X.R., Guo, H.Y., Wu, J.C., Xue, Y.Q., 2004. Effects of water-soluble fractions of diesel oil on the antioxidant defenses of the goldfish, *Carassius auratus* Ecotoxicol. Environ. Saf. 58, 110–116.

Table 1: Dispersant nominal concentration, TPHs and sum of 16 parents and alkylated US-EPA PAHs (ΣPAHs) 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. n.a. = not assessed.

	$[TPHs]_{T=0h}$ (mg/L)	$[TPHs]_{T=48h}$ (mg/L)	$[\Sigma PAHs]_{T=0h}$ (µg/L)	$[\Sigma PAHs]_{T=48h}$ (µg/L)	$[Dispersant]_{nom.}$ (mg/L)
C	n.d.	n.d.	n.d.	n.d.	n.a.
CD	39.1 ± 4.1	25.1 ± 3.1	43.98 ± 5.5	26.34 ± 2.7	3.33
MD	13.15 ± 2.6	9.30 ± 0.2	39.09 ± 0.6	20.63 ± 0.1	n.a.
WSF	n.d.	n.d.	5.16 ± 0.6	0.47 ± 0.07	n.a.
D	n.d.	n.d.	n.d.	n.d.	3.33

Table 2: Concentration of 16 US-EPA PAHs (alkylated and parents) in sea water during CD (Chemically Dispersed oil), MD (Mechanically Dispersed oil) and WSF (Water Soluble Fraction of oil) exposures. Values are expressed as mean ± standard error mean of both experimental replicates. n.d. = not detected.

16 US-EPA PAHs (parents and alkylated)	**Molecular weight (g/mol)**	**Concentration (ng/L) at T=0h and T=48h**					
		T= 0 h			**T=48 h**		
		CD	**MD**	**WSF**	**CD**	**MD**	**WSF**
Naphtalene	128.2	2287±78	1842±101	311±10	335±14	262±6	32±5
C1-Naphtalene	143.2	6936±1699	7569±49	987±22	3244±61	2658±53	78±22
C2-Naphtalene	158.2	15579±199	12766±223	1668±172	7937±393	6396±46	95±9
C3-Naphtalene	173.2	11496±385	9957±59	1298±183	7677±491	6506±47	59±6
C4-Naphtalene	188.2	4488±129	4081±21	450±57	3696±106	3094±226	64±12
Acenaphtylene	152.2	27±3	16±3	n.d.	n.d.	n.d.	n.d.
Acenaphtene	154.2	n.d.	n.d.	n.d.	n.d.	n.d.	1±0
Fluorene	166.2	241±3	196±8	53±9	400±298	89±2	1±0
C1-Fluorene	181.2	336±5	291±4	70±11	663±480	158±2	4±1
C2-Fluorene	196.2	316±3	284±9	44±7	734±523	187±3	3±0
C3-Fluorene	211.2	169±2	130±17	16±1	329±231	57±31	3±0
Phenanthrene	178.2	316±300	522±16	79±4	160±151	241±1	5±0

Anthracene	178.2	3±0	8±8	2±1	n.d.	6±6	n.d.
C1-Phenanthrenes/Anthracene	193.2	959±32	818±17	66±23	569±18	467±3	5±0
C2-Phenanthrenes/Anthracene	208.2	489±4	390±25	34±4	326±2	295±9	n.d.
C3-Phenanthrenes/Anthracene	223.2	136±1	89±0	8±1	75±0	68±2	n.d.
C4-Phenanthrenes/Anthracene	238.2	36±5	29±4	n.d.	23±1	17±3	n.d.
Fluoranthene	202.3	n.d.	2±2	1±0	2±0	1±1	n.d.
Pyrene	202.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C1-Fluoranthenes/Pyrenes	217.3	n.d.	n.d.	n.d.	n.d.	3±3	n.d.
C2-Fluoranthenes/Pyrenes	232.3	n.d.	7±7	n.d.	9±1	5±5	n.d.
C3-Fluoranthenes/Pyrenes	247.3	n.d.	4±4	n.d.	3±3	3±3	n.d.
Benzo[*a*]anthracene	228.3	n.d.	n.d.	n.d.	1±0	n.d.	n.d.
Chrysene	228.3	8±8	9±9	6±3	27±8	19±5	14±2
Benzo[*b*+*k*]fluoranthene	252.3	3±1	6±0	5±0	10±6	8±3	9±1
Benzo[*a*]pyrene	252.3	3±0	3±0	2±0	5±1	5±0	4±1
Benzo[*g*,*h*,*i*]perylene	276.3	34±5	3±3	32±3	4±1	3±1	4±4
Indeno[*1*,*2*,*3*-*cd*]pyrene	276.3	51±31	31±0	3±0	49±0	37±0	40±0
Dibenzo[*a*,*h*]anthracene	278.4	63±3	39±3	25±1	57±4	45±2	48±4

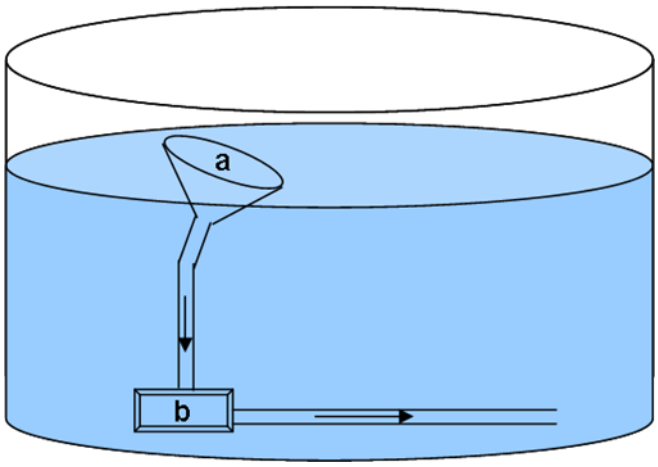

Figure 1: The experimental system constituted of a funnel (**a**) linked to a water pump (**b**) in a 300-l sea tank. (→) indicates the direction of seawater and/or contaminants through the experimental system

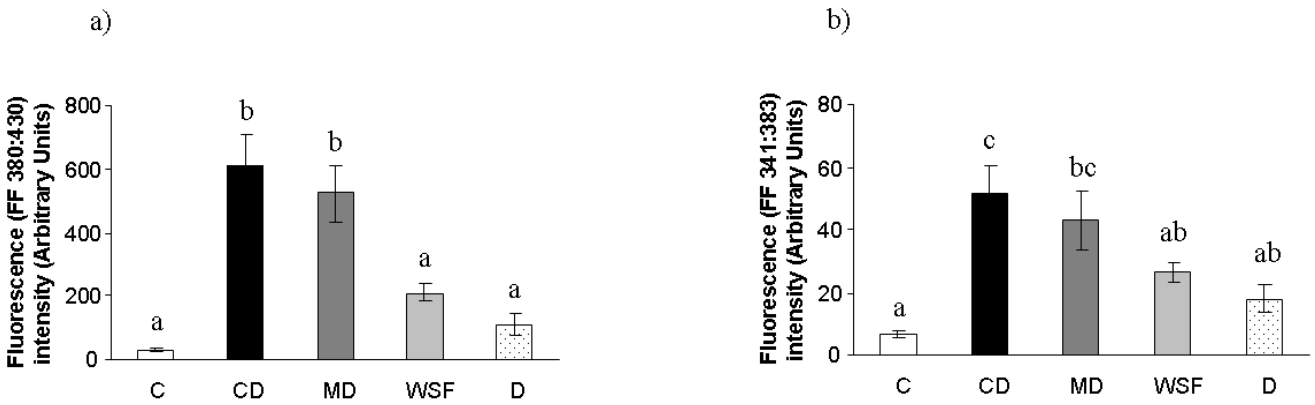

Figure 2: Fixed wavelength fluorescence (FF) of bile reflecting biliary PAHs metabolites levels after 48 h exposure to Control solution (C), Chemically Dispersed oil solution (CD), Mechanically Dispersed oil solution (MD), Water Soluble Fraction solution (WSF) and Dispersant solution (D): (a) FF 380:430 (benzo[*a*]pyrene type of metabolites); (b) FF 341:383 (pyrene derived 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 $P < 0.05$.

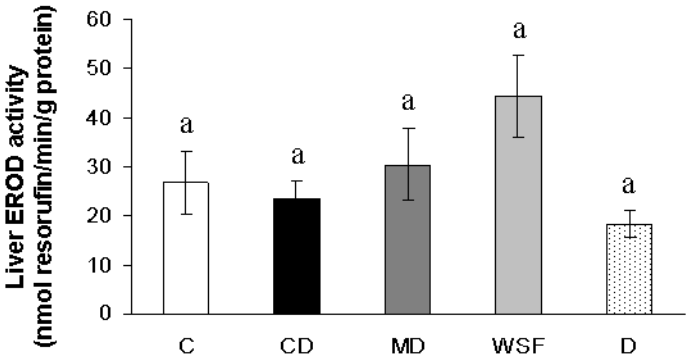

Figure 3: EROD (7-ethoxy-resorufin-O-deethylase) activity in *Liza aurata* after 48 h exposure to Control solution (C), Chemically Dispersed oil solution (CD), Mechanically Dispersed oil solution (MD), Water Soluble Fraction (WSF) solution and Dispersant solution (D). Values represent mean ± standard error (n=10 per treatment). Different letters above bars indicate a significant difference**,** where $P < 0.05$.

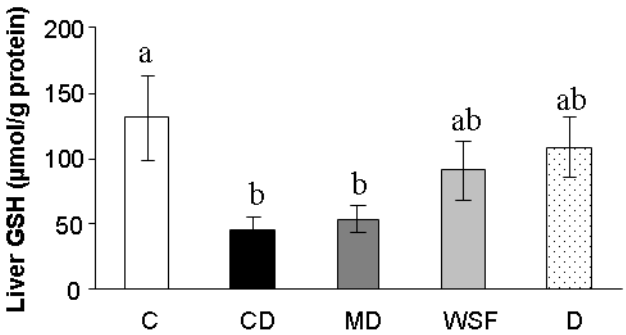

Figure 4: Total glutathione (GSH) content in liver of *Liza aurata* after 48 h exposure to Control solution (C), Chemically Dispersed oil solution (CD), Mechanically Dispersed oil solution (MD), Water Soluble Fraction (WSF) solution and Dispersant solution (D). Values represent mean ± standard error (n=10 per treatment). Different letters above bars indicate a significant difference**,** where $P < 0.05$.

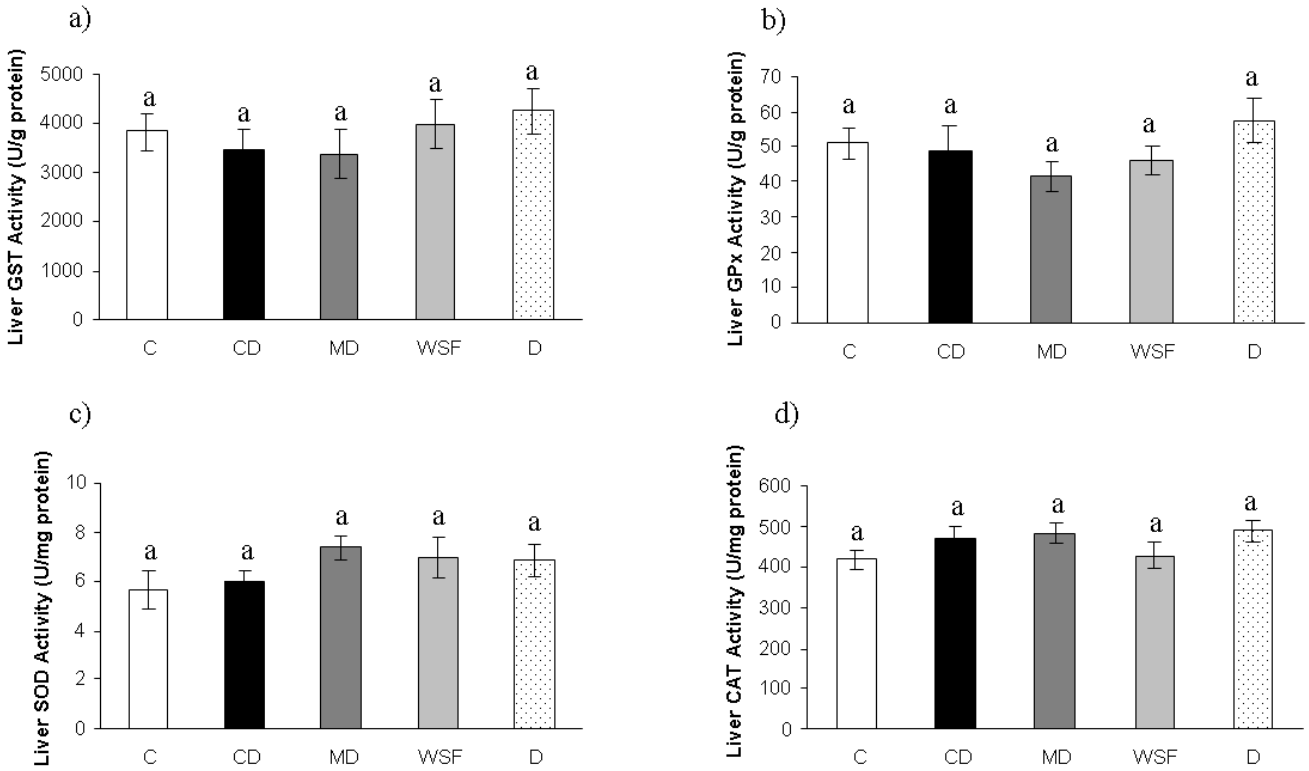

Figure 5: a) Glutathione S-Transferase (GST) activity, b) Glutathione Peroxidase (GPx) activity, c) Superoxide Dismutase (SOD) activity and d) Catalase (CAT) activity in liver of *Liza aurata* after 48 h exposure to Control solution (C), Chemically Dispersed oil solution (CD), Mechanically Dispersed 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 $P < 0.05$.

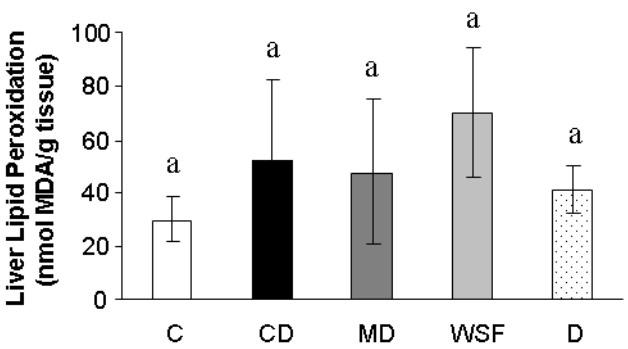

Figure 6: Lipid peroxidation in liver of *Liza aurata* after 48 h exposure to Control solution (C), Chemically Dispersed oil solution (CD), Mechanically Dispersed oil solution (MD), Water Soluble Fraction (WSF) solution and Dispersant solution (D). Values represent mean ± standard error (n=10 per treatment). Different letters above bars indicate a significant difference**,** where $P < 0.05$.

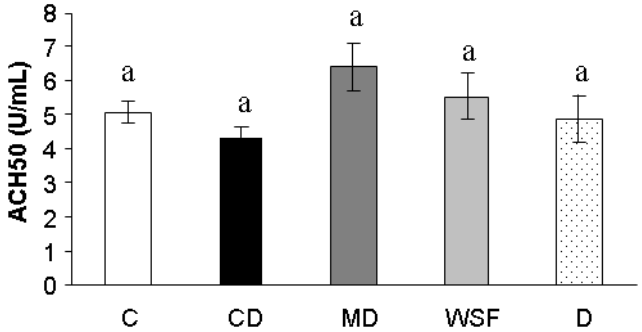

Figure 7: Haemolytic activity of alternative complement pathway (ACH 50) in plasma of *Liza aurata* after 48 h exposure to Control solution (C), Chemically Dispersed oil solution (CD), Mechanically Dispersed oil solution (MD), Water Soluble Fraction (WSF) solution and Dispersant solution (D). Values represent mean ± standard error (n=10 per treatment). Different letters above bars indicate a significant difference**,** where $P < 0.05$.