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Chemical Dispersion of Crude Oil:
Assessment of Physiological, Immune and Anti-oxidant Systems
in Juvenile Turbot (*Scophthalmus maximus*).

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ABSTRACT

This work focuses on the effects of two commercial formulations of dispersants on juvenile turbot after 48h of contamination and 15 days of recovery. Oxidative stress, gill and immune functions were assessed in seven conditions: exposition to the water soluble fraction of an oil, mechanical dispersion, two dispersants alone, two type of chemical dispersion and a control group. In the contaminated groups, nominal concentrations of oil and dispersants were 66 mg.L⁻¹ and 3.3 mg.L⁻¹ respectively.

Dispersants alone had weak effects; the soluble fraction induced leucopenia and gill alteration. Chemical and mechanical dispersion induced similar effects. After contamination, a principal component analysis showed two distinct areas: the first one included the control and dispersants groups, the second one dispersion of the oil. After the 15-day recovery period it was not possible to differentiate the groups.

This study shows that the dispersion, either chemical or mechanical, enhances the consequences of exposure to crude oil without long-lasting consequences.

Keywords:

Chemical dispersant; physiology; immunology; oxidative stress; *Schophthalmus maximus*.

1. Introduction

Chemical dispersion of oil is the main technique available to cope with oil spills at sea and to reduce the impact on marine ecosystems and human populations relying on marine resources. The dispersion process aims at keeping the oil in the water column as a plume of droplets (Canevari 1978), to avoid contamination of shoreline and surface organisms, and to enhance the oil biodegradation (Tiehm 1994; Churchill et al. 1995). The “Net Environmental Benefits Analysis” of this technique suggests that dispersed oil is less harmful for the environment than an oil slick at the sea surface (Lunel et al. 1997). Conversely, in coastal areas where dilution can be restricted due to limited depth and the vicinity of the coastline, dispersant use is often limited or prohibited (Chapman et al. 2007). Nevertheless, in certain cases, the use of dispersants has been shown to be beneficial in coastal sensitive regions (Schuler and Baca 2007). The question of using dispersant in coastal areas, particularly estuaries, remains an unsolved question for responders. Information is needed to evaluate the environmental tradeoffs associated with dispersant use.

Since dispersants potentially decrease exposure for surface and intertidal species while increasing it for water column species, this study focuses on the effects of dispersant use on coastal juvenile fish. Three domains of effect assessment linked with homeostasis were considered: oxidative stress, immune and gill functions.

Oil contamination has been shown to induce a wide range of gill histomorphological alterations (Lopez et al. 1981; Metcalfe 1998; Solangi and Overstreet 1982). As a consequence of these structural impacts, functional impairments can appear. Ionic imbalance has been demonstrated after oil contamination (Kennedy and Farrell 2005; Simonato et al. 2006) possibly in association with a reduction of mitochondria rich cells (MRC; Goanvec et al. 2010). As this cell populations is also directly involved in acid-base regulation in sea water fish (Evans et al. 2005), this study focuses on hydromineral and acid-base equilibrium.

The immune response is of tremendous importance for the health of the organism. It relies upon phagocyte and lymphocyte cells which play a major role in non-specific and specific immune systems (Randelli et al. 2008). Fish immune systems are known to be altered by xenobiotics (Bado-Nilles et al. 2009; Bols et al. 2001; Dunier et al. 1995; Siwicki et al. 2000). PAHs are known to cause cell membrane damage (Ahmad et al. 2003) and to modulate immune cell development (Reynaud and Deschaux 2006). Hence, in this study, WBC counts, leucocyte mortality, complement system and phagocytosis were assessed.

Antioxidant defences are non-specific biomarkers that can also be considered as fish health indicators (Allen and Moore 2004). They demonstrated good responses in the case of oil contamination (Jung et al. 2009; Kopecka-Pilarczyk and Correia 2009; Nahrgang et al. 2009; Oliveira et al. 2008). In this study, antioxidant branchial and hepatic enzyme activities (catalase, superoxide dismutase, glutathione peroxidase) were assessed. Total glutathione, involved in different mechanisms of cellular defence (xenobiotics conjugation and antioxidant defence, van der Oost et al. 2003) was also measured.

The goal of the present study was to assess the relative toxicity of oil chemical dispersion. Turbot (*Scophthalmus maximus*) were exposed for 48h to the water soluble fraction of crude oil, to mechanically dispersed oil, to two dispersants and to the two corresponding chemically dispersed oil. Contamination, stress indicators, hydromineral and acid-base balance, immune parameters and antioxidant enzymatic systems were analysed at the end of the contamination and after two weeks of recovery.

2. Materials and methods

This experiment was conducted in accordance with the European Commission standard 2007/526/EC. The French institute *Cedre* is authorized to conduct experimentation on animals in its capacity as a certified establishment (2006-0429); this experimentation was conducted under the joint responsibility and supervision of Dr. Quentel (habilitation n°:29-008).

2.1. Fish

Juvenile turbot, *Scophthalmus maximus* (n = 140; age: 12 month; mass: 214.2 ± 44.3 g; length: 23.2 ± 1.5 cm; mean \pm SD), were purchased from a fish farm (France Turbot, 22 220 Trédarzec, France). The fish were acclimatized for two weeks in 1200 L tanks supplied with aerated clean seawater ($300 \text{ L}\cdot\text{h}^{-1}$). The lighting regime was set according to the season (April to May). Water salinity (34.0 ± 0.5 ‰), pH (7.78 ± 0.02), oxygen saturation (87.4 ± 2.8 %), and temperature (13.7 ± 0.5 °C) were measured daily (n=25). The fish were fed daily with dried pellets (Le Gouessant®, total protein 54 %, crude fat 12 % of dry weight).

2.2. Crude oil and dispersants.

This study was performed on weathered Arabian Light (WAL) crude oil previously used in other studies (Luna-Acosta et al. 2011; Milinkovitch et al. 2011a). The weathering process consisted in air bubbling through the oil at 12 to 16°C up to a weight loss of 7%. This treatment simulates the 12h ageing of a slick at sea; 12 hours being a minimal delay for an intervention. The WAL contains 54% of saturated hydrocarbons, 34% of aromatic hydrocarbons and 12% of polar compounds (for more detailed composition see Milinkovitch et al. 2011b).

Dispersants were 3rd generation commercial formulations. Dispersant 1 was OSR 62 and dispersant 2 was OD 4000. They were provided by TOTAL Fluids and INOSPEC respectively.

2.3. Experimental schedule and sample collection

The fish (140) were randomly allocated to seven experimental groups: a control group (C), a group exposed to non-dispersed oil (water soluble fraction, WSF), a group exposed to mechanically dispersed WAL (MD), two groups exposed to dispersants alone (d1 and d2) and two groups exposed to chemically dispersed oil (CD1 and CD2). The groups were split into two duplicates (10 fish) and

placed in separate 300 litre exposure tanks. The 14 tanks were equipped with a pumping system allowing continuous water homogenization (fully described by Milinkovitch et al., 2011a). In the case of the WSF condition, the pumping was far enough from the surface to avoid the fractionation of the oil slick. Exposure was started by pouring in 20 g of crude oil for the conditions: WSF, MD, CD1 and CD2, and 1 g of dispersant 1 (conditions d1 and CD1) or dispersant 2 (conditions d2 and CD2). During the exposure period the fish were maintained in closed circulation and then transferred into clean tanks for 15 days of recovery in clean renewed sea water.

Samplings (5 fish per duplicate) were done after the contamination and recovery periods. The fish feeding was stopped 48h before sampling. Blood (2ml) was collected from the caudal vein with lithium heparinized vacutainer (BD Vacutainer™ LH 85 U.I.). 1,6 ml of blood was sampled for acid-base, hydro-mineral equilibrium, glucose and WBC analysis. The remaining blood was centrifuged (1200g, 10 min, 4°C) and plasma samples stored at – 80°C before analysis. The fish were then killed, weighed and measured. Liver, spleen, gill arches and gall-bladder were sampled, weighed and stored at -80°C.

2.4. Analytical methods.

Contaminant concentration

The total petroleum hydrocarbon concentration (dissolved hydrocarbons and oil droplets) was measured in duplicate at the beginning and at the end of the contamination period. One hundred ml samples were extracted 3 times with 10 mL of dichloromethane (Carlo Erba Reactifs, SDS). The combined organic phases were dried on anhydrous sulphate and absorbance was measured at 390 nm (UVeVis spectrophotometer, Unicam, France) as described by Fusey and Oudot (1976). Results are expressed in mg.L^{-1} , and the linearity of the response was checked between 1 and 100 mg.L^{-1} .

Biliary fluorescence

Fluorescence analyses of bile samples were performed with a Jasco FP-6200 (Tokyo, Japan) with 5 nm slit widths on emission and excitation channels, as described by Aas et al. (2000). The

excitation and emission wavelengths used were 343–383 (for four-ringed PAH compounds) and 380–430 nm (characteristic of the benzo[a]pyrene-type metabolites) (Aas et al. 2000; Krahn et al. 1984, 1986; Lin et al. 1996).

Stress & metabolism

Glucose concentration in blood was determined by a clinical i-STAT analyzer using EC4+ cartridges (i-STAT corporation, Windsor, USA) (Foss et al. 2004; Petri et al. 2006). Cortisol and lactate levels were measured using commercial kits from the Cayman chemical company (USA) and BioVision Research products (USA) respectively.

Plasma ions and osmolality

All blood parameters were measured (in duplicate) on plasma samples stored frozen until analysed: osmolality was measured on a VAPRO® vapor pressure osmometer (model 5520 WESCOR); plasmatic chloride and sodium concentrations were respectively measured by argentimetric titration with a chloridometer (CORNING Chloride analyzer 925), and on a flame photometer (IL 243-05).

Acid-base parameters

Blood acid-base characteristics were determined from aliquots of about 500 μ l each as described by Maxime et al. (2000). In brief, blood pH was measured by using a Fisher Scientific glass microelectrode; the partial pressure of carbon dioxide in arterial blood ($p\text{CO}_2$) was determined by the Astrup method (Astrup 1956). The bicarbonate concentration in arterial blood was calculated from the Henderson-Hasselbalch equation, using a CO_2 solubility coefficient and an operational pK' from Boutilier et al. (1985).

White blood cells:

Total white blood cell counts (WBCC) and differential leucocyte counts were carried out according to Quentel and Obach (1992). WBCC's were conducted employing a Thoma haemocytometer and Kekic and Ivanc's method (1982) using Giemsa solution as diluents (1/200) and stainers. Differential leucocyte counts were made from blood smears stained with May-Grunwald Giemsa. A total of 100 leucocytes were counted from each smear to obtain the percentage of the different types of leucocytes. Lymphocytes, neutrophil granulocytes and monocytes were identified using standard morphological criteria according to the description given by Burrows and Fletcher (1987). Numbers of granulocytes and lymphocytes were then estimated from the WBCC.

Leucocyte mortality and phagocytic capacity were carried out with a Facscalibur flow cytometer (Becton Dickinson) according to Bado-Nilles et al. (2009). Mononuclear cells were collected after centrifugation on a Ficoll gradient (400 g, 30 min, 15°C, and density of 1.07 to 1.08 g.cm⁻³). Cell numerations were performed with Thoma's cell haemocytometer and adjusted at 10⁶ cell.mL⁻¹ with L15 medium. For each cell sample, 10 000 events were counted and analyses were made on all immune cells. Cell mortality was determined using 300 µL of leucocyte suspensions and 15 µL of Propidium iodide (PI, 1.0 g.L⁻¹, Molecular Probes) after an incubation period of 30 min at 4°C. Phagocytic capacity was analyzed on 300 µL of leucocyte samples and 15 µL of a 1/10 dilution of fluorescence beads (2.7 x 10¹⁰ particules.ml⁻¹, Fluorosphere® carboxylate-modified microspheres, diameter 1 µm, Molecular Probes) after 1 hour of incubation at room temperature. The fluorescence setting was established using a suspension of fluorescence beads in distilled water and only the events showing a fluorescence of at least three beads were considered positive for phagocytosis activity.

Plasmatic immune parameters

Plasma lysozyme activity was determined using a turbidimetric assay (Grinde et al. 1988), adapted to microtitration plates (Desvignes et al. 2002) and to turbot species. Briefly, a bacterial suspension of *Micrococcus lysodeikticus* (Sigma) was prepared at a concentration of 1.25 g.L⁻¹ (in a

0.05 M sodium-phosphate buffer pH 6.2). Fifty micro-liters of plasma were plated in 96 well microtitration plates. The reaction was initiated in a multiscan spectrophotometer, by addition of 160 $\mu\text{L}\cdot\text{well}^{-1}$ of *M. lysodeikticus* suspension using an automatic dispenser. Reading of D.O. at a wavelength of 450 nm was performed every 15 s for 3 min, the plate being shaken before each reading. Using a standard hen egg white lysozyme (Sigma) in a sodium-phosphate buffer, the concentration of lysozyme in turbot plasma was expressed in $\text{mg}\cdot\text{L}^{-1}$.

Determination of the alternative pathway of plasma complement activity was carried out at 4 °C through a haemolytic assay with rabbit red blood cells (RRC, Biomérieux) as described by (Yano, 1992) and adapted to microtitration plates. Samples, diluted to 1/32 in a EGTA–Mg–GVB buffer to avoid natural haemolytic activity, were added in increasing amounts, from 10 to 100 $\mu\text{L well}^{-1}$. The round-bottomed wells were then filled with a EGTA–Mg–GVB buffer to a final volume of 100 μL and 50 μL of 2 % RRC suspension was added to all the wells. Control values of 0% and 100 % haemolysis were obtained using, respectively 100 μL of EGTA–Mg–GVB buffer and 100 μL of non-decomplemented trout haemolytic serum (diluted at 1/50 in ultrapure water). Samples were incubated for 1 h at 20 °C. The microplates were centrifuged (400-g, 5 min, 4 °C). Then, 75 μL of supernatant from each well were transferred with 75 μL of phosphate buffered saline (PBS, Biomérieux) into 96 flat-bottom microplate wells. The absorbance (A_{540}) was read in a Labsystems' iEMS analyser and the number of ACH_{50} units per mL of plasma was determined by reference to the 50 % haemolysis using the formula established by Yano (1992).

Anti oxidant defense systems in gills and liver

Liver and gills were homogenized in a phosphate buffer (PO_4 Buffer 100mM, glycerol 3.1%, phenylmethanesulfonyl fluoride 0.2 mM, pH 7.8) using an ultraturax (polytron®). The resulting homogenate was centrifuged at 10,000 g for 15 min at 4 °C. The supernatants collected were used for protein estimation and oxidative stress biomarkers.

Before enzymatic assays, protein content was estimated spectrophotometrically by the Bradford method (Bradford 1976) adapted to microplate using bovine serum albumin as a standard. Enzyme activities were then expressed as enzymatic units per total protein content ($\text{U}\cdot\text{mg}^{-1}$ total protein).

Superoxide dismutase (SOD) activity was determined by the method of Paoletti et al. (1986). This assay is based on the inhibition of NADH oxidation by the superoxide dismutase. Superoxide dismutase activity was assessed spectrophotometrically at 340 nm and expressed as the amount of enzyme/mg of protein. The catalase (CAT) activity assay, using the spectrophotometric measurement of H_2O_2 breakdown, measured at 240 nm, was performed following the method of Beers and Sizer (1952). Glutathione peroxidase (GPx) activity was assayed following the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase according to the method of Paglia and Valentine 1967. One unit of CAT or GPx activity is defined as the amount of the enzyme that consumes 1 μmol of substrate or generates 1 μmol of product per min. Glutathione level was assayed using the methods described by Vandeputte et al 1994. 5,5-Dithiobis-(2-nitrobenzoic acid) (DTNB) is a disulfide chromogen and turns dark yellow when reduced with sulphhydryl compounds. The absorbance of reduced chromogen was followed at 412 nm. GSH content was determined from a standard.

2.5. Statistical analyses.

Statistical tests were performed using Statistica (9.0, Statsoft). In order to compare the evolution of the physiological status of the fish according to the contaminants:

1/ For every parameter measured, Lilliefors' and Bartlett's tests were used to check normality and homocedasticity. ANOVA or Kruskal–Wallis analyses were then performed and if necessary were followed by Fisher tests.

2/ Two matrixes were prepared considering the variables showing significant changes in the experimental conditions tested \times 140 individuals after contamination and after decontamination. These matrixes were analysed using Principal Component Analysis (PCA).

3. Results

3.1 Total petroleum hydrocarbon

TPH are given in table 1. In the case of WSF, TPH concentration was close to 7 mg.L⁻¹. The MD induced, when compared to the WSF, a 6 fold increase of TPH concentration. The use of the dispersants results in a larger increase of TPH concentration (of 10 fold, close to 70mg.L⁻¹). In d1 and d2 conditions, where the dispersants were present without crude oil, TPH ranged between 3 to 5 mg.L⁻¹. In the control group and during the decontamination period, TPH concentrations were not detectable.

3.2 General state of the fish

During the whole experiment, no mortality was recorded and no external nor internal lesions were observed. Table 2 shows the different condition indexes calculated after contamination and recovery. The different treatments did not induce any significant modification of Fulton's condition index (K), nor of hepato-somatic index (HSI). After contamination, the spleno-somatic index (SSI) in the condition CD1 was lower than in the condition d2. This effect was no longer present after decontamination.

3.3 Effects of experimental conditions

Parameters linked to contamination, stress and metabolism, blood homeostasis, immune function and anti-oxidant systems were measured after 48 hours of contamination and after 15 days of recovery. Tables 3 and 4 synthesise the results obtained and the conclusions of the statistical tests.

Water Soluble Fraction exposure (WSF)

After contamination in the WSF condition, fixed fluorescence at 343-383 nm showed a significant increase of 3.8 times the control group value (Table 3); the response at 380: 430 nm was not significant. After recovery (Table 4), all responses were very low and there was no longer a difference between C and WSF groups.

WSF contamination induced alterations of hydromineral and acid-base balances, haematological and anti-oxidant parameters after contamination (table 3). The plasmatic osmolality was increased from 300 to 311 mOsm (table 4), and $[\text{HCO}_3^-]$ rose from 5.4 to 7.6 mM without blood pH change (Figure 1). Leucocyte and granulocyte counts were greatly reduced by the contamination: their numbers were respectively divided by 2.5 and nearly 12 when compared to the control. Amongst the different parameters linked to enzymatic anti-oxidant systems, only the GSht in the gills was significantly increased.

After 15 days of recovery in clear water, all parameters were similar to the control values and no significant effects were observed (Table 4).

Mechanical Dispersion (MD)

Bile fluorescence showed a marked increase for MD when compared to the control and WSF: both OH-pyrene (343:383 nm) and OH-BaP (380:430 nm) type metabolites were increased (Table 3). After recovery (Table 4) the contamination was no longer detectable.

MD induced modifications of hydromineral, acid-base balance and WBC counts (Table 3). Plasmatic osmolality, sodium and chloride ions concentration were significantly increased after the contamination. Figure 1 shows different acid-base alterations in MD and WSF contaminations: in the MD case, blood pH and pCO_2 were modified without change in $[\text{HCO}_3^-]$. The modifications of haematological parameters were similar to those observed in the case of WSF: a marked reduction in leucocyte numbers and a fall in the granulocytes. No significant response could be observed (when compared to the controls) in neither immune nor anti-oxidant parameters.

After recovery hydromineral and acid-base equilibrium were no longer different from the control group. In contrast, leucocyte and lymphocyte numbers remained lower.

Dispersants (d1 & d2)

Exposure to d1 and d2 did not induce any modification of bile fluorescence (Table 3). Consistently, there was no response of biliary fluorescence after recovery (Table 4).

D1 had no effect on the different parameters analysed, except an increase in the plasmatic chloride concentration after contamination. D2 had the same effect on $[Cl^-]$ but induced also: a slight decrease in blood pH, a 50% increase in leucocyte numbers and a 1.7* increase in GSht in the gills (Table 3).

None of the parameters measured was modified after the recovery period (Table 4).

Chemical Dispersion (CD1 & CD2)

CD1 and CD2 induced similar increases of bile fluorescence (Table 3). In the case of OH-pyrene type metabolites (343:383 nm) the values obtained were close (71.6 and 68 AU) and statistically above the mechanical dispersion (53.4 AU), the WSF (27.5 AU) and the oil-free conditions (C, d1 and d2). In the case of OH-BaP type metabolites (380:430 nm) the fluorescence values were not significantly different from the MD group but higher than the C, WSF d1 and d2 groups. After the recovery, there was no longer any difference of bile fluorescence (Table 4).

The parameters related to stress and metabolism, immune parameters and antioxidant systems showed no difference between CD1 and CD2 and were not different from the control values after contamination. CD1 had no effect on the hydromineral equilibrium while CD2 induced significant increases in plasmatic osmolality and chloride ion concentration (when compared to C). Acid-base homeostasis was modified by both treatments but in different ways: CD1 lead to a rise in $[HCO_3^-]$

without change in blood pH and pCO₂, and CD2 induced opposite alterations: significant pH and PCO₂ changes without [HCO₃⁻] modification (see Figure 1). WBC counts were modified as in the case of WSF and MD: leucocyte and granulocyte numbers were severely reduced; in the case of CD1 the lymphocyte number also fell significantly.

After recovery, leucocyte numbers were still lower than in the controls and lymphocyte counts showed a decrease in the case of CD2 group. Considering the anti-oxidant systems, CD1 induced a 50% fall of catalase activity in the liver. Hydromineral balance and acid-base equilibrium were normalised and there was no difference between control and CD1 and CD2 results for any other parameters.

3.4 Integrated Approach

Principal component analysis (PCA) has been used in order to fulfil two goals: 1/ to establish (independently from the experimental contamination) possible correlations between the level of contamination and the other parameters analysed; 2/ to have a global overview making it possible to compare the effects of the experimental contaminations.

Amongst the twenty six parameters studied, the statistical analyses exposed in Tables 3 and 4 shows that plasmatic glucose concentration, phagocytosis, ACH50 and lysosyme activity were not statistically modified in any experimental condition at any time. Consequently these parameters were not integrated in the subsequent analysis (PCA).

Correlation Level of Contamination and Biological Parameters

In order to analyse the correlation between the biliary fluorescence (reflecting the contamination level) and the other parameters, a first Principal Component Analysis was made of the results obtained after the contamination period (PCA 48h). The three main factorial axes explained 22.22%, 13.12%

and 9.90% of the total variance, respectively. The projection of the quantitative variables of the first matrix (48h of contamination) onto the correlation circle shows:

1/ a moderate positive correlation of biliary fluorescence with plasmatic cortisol (figure 2 b), hydromineral equilibrium parameters (plasmatic osmolality, chloride and sodium concentrations) and acid-base equilibrium (plasmatic concentration in HCO_3^- and pCO_2 ; figure 2 a & b).

2/ a strong negative correlation between the contamination level and leucocyte numbers (leucocytes, granulocytes and lymphocytes, figure 2 a), and with the anti-oxidant enzymatic systems especially in the gills (figure 2 b).

General Overview of Experimental Contaminations

Considering the distribution of the fish over the factorial plan (axis 1×axis 2), PCA48h shows two distinct zones (Figure 3 a): the first one is composed of the three control groups (C, d1 and d2) and the second one of the dispersed groups (MD, CD1 and CD2). The fish exposed to WSF are in an intermediate position. In the same projection of the individuals onto the factorial axis after 15 days of recovery in clear water (PCA15d, Figure 3 b) it is no longer possible to differentiate the experimental conditions.

4. Discussion

In this experiment, juvenile coastal turbot (*Schophthalmus maximus*) were exposed for 48h to the water soluble fraction of crude oil, to mechanically dispersed oil, to two dispersants and to the two corresponding chemical dispersion. Contamination levels, general stress indicators, hydromineral and acid-base balance, immune parameters and antioxidant enzymatic systems in gills and liver were

analysed at the end of the 48h contamination and after two weeks of recovery in clean sea water in order to analyse the relative toxicity of dispersed oil in an accidental context.

Contamination Setup

The results show detectable levels of hydrocarbons in the d1 & d2 conditions. This can be due to the presence of hydrocarbon solvents in the dispersants. These saturated hydrocarbons facilitate the diffusion of the surfactants through the oil slick to the interface oil/water and enhance the efficiency of the dispersants (Lessard and Demarco 2000). In the case of the WSF, the contamination is roughly 10 times lower than the nominal TPH (7.2 mg.L^{-1}). The diffusion from the oil is slow and the final concentration is the result of the competition between this diffusion and the evaporation of the more volatile molecules from the oil slick. When the oil is mechanically dispersed, the TPH is much higher (44.5 mg.L^{-1}). The mechanical energy allows the formation of oil droplets without preventing oil adherence on the experimental system. In the case of the chemical dispersion, the measured concentrations are very close to the nominal concentration, showing that the dispersants make the formation of stable non-adhering droplets possible.

These concentrations have to be compared with in-situ analyses of spills at sea. Observations of the fate of the oil after dispersant use show increases of TPH in the top ten meters up to 20 to 60 mg.L^{-1} (Lessard and Demarco 2000; Lewis et al. 1998; Lunel et al. 1997; Spooner 1970). Wave dispersion can have similar effects as demonstrated by the T. Braer oil spill where 86 000t of crude oil were mechanically dispersed by severe meteorological conditions. In this case, TPH concentrations reported by Lunel (1995) ranged from 1 to 100 mg.L^{-1} . These data show that the contamination conditions obtained here can be considered as representative of dispersant use in the environment.

The General State and Stress Response of the Fish

In fish, exposure to toxic products can lead to the invasion of environmental pathogens, the appearance of opportunistic infections (Grinwis et al. 2000), and the alteration of condition indexes (van der Oost et al. 2003). In this study, neither mortality, nor external / internal lesions were noticed. These results are in agreement with the study of Milinkovitch et al. (2011a) performed with a similar experimental setup on juvenile *Liza aurata*. Dispersants, when used alone tended to increase SSI (considered as a general indicator of overall immune system competence). However no statistical difference was observed with the controls and only the SSI of d2 fish was significantly higher than that of CD2 condition. The relatively short exposure time and the controlled conditions in the experimental system (filtered and UV-sterilized water, constant temperature and high oxygenation) probably prevented any impact of the different treatments upon the general health of the fish.

Cortisol, glucose and lactate concentration modifications can provide evidence of the activation of the hypothalamic-pituitary-interrenal axis and acute energy mobilization to cope with homeostasis threat (Bonga 1997; Mommsen et al. 1999). At the end of the contamination period, glucose concentration remained stable whatever the treatments. Although WSF seemed to increase cortisol and lactate (Table 3) and the existence of a weak correlation (Figure 3b) between contamination levels and cortisol (suggesting a stress response) no significant difference of these two parameters was recorded when compared to controls. Surprisingly, with dispersants alone, mechanical and chemical dispersion decreased cortisol or lactate plasma concentration when compared to WSF. These results are not in accordance with the work of Kennedy and Farrell (2005) on the Pacific herring where a stress response was observed with lower TPH concentrations. Two points could explain this difference. First, the contamination of the Pacific herring was obtained flowing sea water through a column of oil-coated beads favoring the presence of low molecular weight PAH. In the present study these compounds are partly evaporated during the weathering process. Then, the maximal stress response of the Pacific herring was observed between 4 to 24h and then decreased rapidly. Here, the first sampling after 48h of contamination could occur after the stress response of the fish.

Fish Contamination

In fish, hydrocarbons will reach the internal medium principally via the gill epithelium (Randall et al. 1998). Dispersion of oil may increase contact with and uptake of hydrocarbons. Here, individual contamination levels were estimated measuring biliary fluorescence, a classical biomarker of exposure to petroleum compounds in fish (Aas et al. 2000; Jung et al. 2009). It makes a reliable and rapid evaluation of fish PAH contamination possible.

The results of OH-pyrene metabolites are in good coherence with the TPH concentrations in the water and clearly show that (i) WAL dispersion increases the availability of the contaminant: (WSF fluorescence is lower than those of the three dispersed conditions); (ii) hydrocarbon contamination is further increased by CD (MD is lower than CD1 and CD2 fluorescence). This result confirms the work of Ramachandran et al. (2004) where chemical dispersant had been shown to enhance the uptake of PAH in trout.

Hydromineral and Acid-Base Equilibriums

Gills are multitasking organs, involved in a number of physiological functions (Evans, 2008; Evans et al. 2005). In the present work, since oil is known to have structural and functional impacts on gills (Metcalf 1998; Kennedy and Farrell 2005; Simonato et al. 2006), hydromineral and acid-base equilibrium have been used to assess gill-based homeostasis.

WSF and MD results confirm previous work on the effect of oil or PAH exposure upon ionic balance in marine fish (Goanvec et al. 2010) where short-term contamination leads to increases in plasmatic ion concentration eventually associated with an MRC reduction. These observations are in coherence with the positive correlation between the parameters used to assess the hydromineral equilibrium (Osm, Cl^- , Na^+) and the contamination levels (see figure 2 a & b). More interesting are the effects observed with dispersants alone and the results of chemically dispersed oil. The d conditions modified chloride concentration; the effects of CD2 were close to the effects of MD with an osmotic

imbalance and an increase of chloride ions, but surprisingly CD1 did not induce any change in hydromineral equilibrium. The literature focussing on the effects of dispersants on ionic balance being scarce, it is difficult to propose a hypothesis explaining these results. Nevertheless, it worth noticing that Duarte et al. (2010) working on juvenile tambaqui (a tropical freshwater fish) did not notice any change of ionic plasma during a 24h exposure to dispersant alone, but they provide evidence of a clear ionic imbalance in the case of dispersant and crude oil during the first 12 hours of exposure followed by restoration after 24 hours. The differences observed here can eventually be due to interspecific differences or to the rapid kinetics of hydromineral equilibrium alteration and recovery that were not observed here.

The acid-base equilibrium observed here is typical of teleosts where the high specific ventilation and CO₂ solubility lead to low blood pCO₂ and bicarbonates and relatively alkaline pH (Heisler, 1986). In the control group pH values are nevertheless slightly acidic when compared with data obtained in similar conditions on turbot (Maxime et al. 2000) presumably as a consequence of the water pH. The results presented on a Davenport diagram (figure 1) are typical of a respiratory acidosis (for the MD and CD2 groups) compensated by a metabolic alkalosis (in the case of WSF and CD1 exposure). The respiratory acidosis is the direct consequence of a CO₂ accumulation inducing an increase of blood H⁺ and HCO₃⁻ concentration. In water breathers, metabolic compensation is mainly due to proton secretion in sea water and bicarbonate release in the blood stream from gill MRC (Perry and Gilmour, 2006). Setting aside the question of dispersants, the respiratory acidosis could be the consequence of a structural alteration of the gill epithelium that can appear with oil contamination (Haensly et al. 1982; Khan 1999), which could reduce gas exchanges and increase pCO₂. On the other hand, the absence of compensation observed for MD suggests an MRC impairment in the case of the mechanical dispersion (as observed by Goanvec et al. 2010). Dispersant 2 had a slight effect on its own leading to a moderate increase in pCO₂. This result could be consistent with the observations of Khan and Payne (2005) who reported that the dispersant Corexit 9527 can induce structural alterations of gill epithelium in seawater fish. Interestingly, d1 does not only have no effect when used alone but seems also to have a protective effect when associated with crude oil (as was observed for

hydromineral balance) since CD1 shows a metabolic compensation. Further investigations are clearly necessary to clarify the mechanisms of effects of crude oil and dispersants on these homeostatic functions and to verify the hypothesis of an alteration of MRC's.

Hematology and Immune Parameters

Variations of blood cellular composition are reported among the first disturbances described in organisms exposed to environmental constraints (Reynaud and Deschaux 2006). Here, a decrease in WBC due to a granulopenia and, to a lesser extent to a lymphopenia, was observed in all oil exposed turbot (i.e. WSF, MD, CD1, CD2). Furthermore, PCA analysis showed a negative correlation between the contamination level and the WBC counts. This leucopenia thus seems to be due to hydrocarbons. Moreover leucocytes did not exhibit higher mortality in oil-exposed fish in spite of direct hydrocarbon cytotoxicity (Schirmer et al. 1998). This result does not agree with previous studies in sea bass where an increase of leucocyte mortality was reported following a 7 day exposure to the soluble fraction of heavy fuel oil (Bado-Nilles et al. 2009), or a 21 day exposure to crude oil (Danion et al. 2011a). In this study, it could be proposed that the exposure time is too short to observe cellular membrane damage. After fifteen days of recovery the leucopenia was still noted in fish exposed to dispersed oil (either mechanically or chemically). This deeper damage to lymphocyte cells in dispersed groups could be due to the higher contamination level. A similar lymphopenia was previously observed by Danion et al. (2011b) in sea bass exposed for 48 hours to a high concentration of WSF of Arabian crude oil.

In spite of the fall of leucocytes, no impact on the immune parameters was reported. The plasmatic parameters were not modified by any experimental condition. The phagocytosis was not affected either. As for the leucocyte mortality, the short exposure time could explain this lack of response.

Antioxidant Systems

PAH exposure is known to generate reactive oxygen species (ROS) (Jifa et al. 2006). SOD is a primary scavenger of ROS (Kappus 1985), and further steps involve GPx and CAT. Protective and adaptive roles of glutathione against ROS toxicity are also well established (Oliveira et al. 2008; Saera-Vila et al. 2009).

Concerning enzymatic antioxidant defenses and glutathione, the PCA analysis makes it possible to establish a negative correlation between the contamination level and GSHt & GPx in gills and liver. But surprisingly, GSHt analysis shows that d2, WSF and CD2 increase the total glutathione content in the gills or liver. It is known that biomarker responses can be influenced by a large number of confounding factors (Martinez-Alvarez et al. 2005) that can make their analysis difficult. In particular, environmental contaminants can induce enhancement (Gallagher et al. 1992 ;Thomas and Juedes 1992; Thomas and Wofford 1984) or inhibition (Milinkovitch et al. 2011b; Zhang et al. 2004) of glutathione synthesis. In this experiment this could lead to opposite effects. And, if the d2 and low levels of contamination seem to induce an increase in the antioxidant response; higher concentration of contaminants (MD and CD) impair this adaptive process leading to the global negative correlation between contamination and antioxidant defences. Further experiments on the dose-response relationship would be necessary to confirm this hypothesis and determine the mechanisms underlying these results.

5. Conclusion

Amongst the different parameters evaluated in this study, the main results came from gill-based homeostasis and hematology. The Arabian light oil induced a marked leucopenia and an alteration of hydromineral and acid-base equilibrium, the mechanical dispersion leading simply to more pronounced effects. When used alone, the two commercial dispersants appeared to have only weak consequences (rise in leucocyte numbers and of plasmatic Cl^- and H^+ concentrations). The chemical dispersion led to comparable effects to those of the mechanical dispersion, but CD1 induced important changes in hematological parameters while CD2 elicited more pronounced effects on gill-

based homeostasis. The principal component analysis clearly synthesizes these results; the distribution of the fish shows, after the 48h contamination period (figure 3a), two distinct areas on the factorial plan. The first one comprises the control group and the two dispersants; the second one includes the three different types of dispersion (mechanical and chemical) of the Arabian light oil. The non-dispersed oil (WSF) is placed in an intermediate position, overlapping with the two previous areas. After 15 days of recovery it is no longer possible to differentiate any group, all the fish are situated in the same zone of the plan (figure 3b).

The goal of this study was to assess the relative toxicity of chemical dispersion of oil in a coastal context. Our study clearly shows that on juvenile turbot, the dispersion (either mechanical or chemical) enhances the consequences of exposure to oil, but apparently without long-lasting consequences. Nevertheless, it worth noticing that the decision to use dispersant should take into account the more vulnerable biological resources of the area of interest at the time of the spill. It is a limit of the present work that should be followed by a study of the effects on early life stages (gametes, eggs and larvae) known to be very sensitive to dissolved oil compounds.

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Table 1
Total petroleum hydrocarbon in sea water during the contamination.

	[TPH] (mg.L ⁻¹)	Nominal oil concentration (mg.L ⁻¹)	Nominal dispersant concentration (mg.L ⁻¹)
C	n.d.	-	-
WSF	7.2 ± 0.6	66.6	-
MD	44.5 ± 3.1	66.6	-
d1	5.2 ± 3.9	-	3.3
d2	3.3 ± 2.6	-	3.3
CD1	68.7 ± 3.2	66.6	3.3
CD2	71.5 ± 5.2	66.6	3.3

TPH (mean ± sem, n= 4), oil and dispersant nominal concentration in the seven exposure. C: Control, WSF: Water Soluble Fraction of oil, MD: Mechanical Dispersion, d1 & d2: dispersants, CD1 & CD2: Chemically Dispersed oil.

Table 2: Conditions indexes after contamination and recovery period.

		C	WSF	MD	d1	d2	CD1	CD2
48 hours of contamination	K	1.73	1.65	1.66	1.69	1.66	1.72	1.68
		0.06	0.05	0.04	0.02	0.04	0.06	0.04
	HSI	0.89	0.86	0.90	0.85	1.01	0.92	1.12
		0.06	0.03	0.06	0.04	0.04	0.05	0.13
	SSI	0.09^{ab}	0.07^{ab}	0.07^{ab}	0.10^{ab}	0.13^b	0.06^a	0.08^{ab}
		0.01	0.01	0.01	0.01	0.02	0.01	0.01
15 days of recovery	K	1.65	1.65	1.63	1.78	1.73	1.75	1.66
		0.02	0.04	0.03	0.06	0.04	0.05	0.04
	HSI	1.17	1.26	1.13	1.30	1.03	1.25	1.21
		0.06	0.08	0.07	0.13	0.05	0.06	0.07
	SSI	0.10	0.09	0.09	0.10	0.09	0.08	0.09
		0.01	0.01	0.02	0.02	0.01	0.01	0.01

K : Fulton's condition index, HSI: hepato-somatic index, SSI: spleno-somatic index, C: Control group, WSF: Water Soluble Fraction of oil, MD: Mechanical Dispersion, d1 & d2: dispersants, CD1 & CD2: Chemically Dispersed oil. Mean values \pm sem. n=10. Letters indicate significant differences between groups (Kruskall Wallis tests)

Table 3 : Contamination level, stress and metabolism, blood homeostasis, immune function and anti-oxidant systems after 48 hours of contamination.

		C	WSF	MD	d1	d2	CD1	CD2
biliary fluo.	343:383	6.99^a	27.51^b	53.42^c	7.33^a	5.29^a	71.64^d	67.97^d
	(ANOVA)	0.42	3.53	4.24	0.42	0.65	5.42	5.95
	380:430	5.02^a	4.99^a	7.69^b	4.37^a	3.19^a	9.02^b	8.59^b
	(ANOVA)	0.63	0.46	0.86	0.28	0.44	0.59	0.79
stress indicators	Cortisol	27.1^{ab}	40.3^a	18.0^b	20.3^b	12.6^b	27.5^{ab}	15.9^b
	(ANOVA)	4.7	7.0	2.4	3.4	2.8	4.4	4.0
	Glucose	25.6	23.8	25.1	23.6	26.1	24.2	30.3
	(KW)	1.2	1.4	1.3	1.1	0.9	1.0	1.8
	Lactate	27.9^{ab}	48.8^a	22.8^{ab}	24.1^{ab}	35.4^{ab}	17.1^b	25.6^{ab}
	(KW)	5.0	10.2	2.9	3.4	12.9	2.0	7.7
hydromineral balance	Osm	299.7^a	311.4^b	312.2^b	307.3^{ab}	305.9^{ab}	307.2^{ab}	311.3^b
	(ANOVA)	2.8	2.6	3.6	3.8	6.0	2.6	1.9
	[Na+]	146.6^a	151.1^{ab}	158.2^b	145.9^a	145.1^a	151.5^{ab}	151.9^{ab}
	(ANOVA)	2.5	2.5	3.9	1.7	5.1	3.3	3.2
	[Cl-]	106.1^a	107.9^{ab}	111.1^b	112.3^b	112.4^b	109.8^{ab}	111.4^b
	(ANOVA)	1.1	1.4	1.5	2.2	1.5	1.8	2.1
acid-base balance	pH	7.68^a	7.70^a	7.56^b	7.68^{ac}	7.63^c	7.70^a	7.55^b
	(ANOVA)	0.03	0.02	0.03	0.03	0.02	0.01	0.02
	[HCO3-]	5.37^a	7.61^b	6.44^{ab}	5.62^a	6.32^{ab}	7.71^b	6.28^{ab}
	(ANOVA)	0.33	0.32	0.24	0.98	0.74	0.33	0.44
	pCO2	3.86^a	4.73^a	5.68^b	3.43^a	4.64^a	4.79^a	5.44^b
	(ANOVA)	0.39	0.30	0.44	0.60	0.56	0.29	0.33
hematology	leucocytes	74.4^a	29.4^b	16.3^b	85.3^{ac}	111.9^c	10.9^b	25.3^b
	(ANOVA)	8.3	7.9	2.8	11.6	18.6	2.5	5.8
	granulocytes	26.1^a	2.2^b	2.0^b	27.8^a	19.4^a	3.0^b	2.3^b
	(KW)	6.9	0.6	0.4	5.7	3.1	1.0	0.9
	lymphocytes	48.3^{ab}	27.2^{abc}	14.3^{bc}	57.5^a	92.5^a	7.9^c	23.0^{abc}
	(KW)	8.5	8.0	2.6	7.7	18.8	2.3	5.0
	leu. mortality	1.07	0.82	0.54	0.64	0.99	0.64	0.50
	(KW)	0.26	0.23	0.09	0.18	0.21	0.18	0.06
immune parameters	phagocytosis	25.2	15.0	11.9	22.1	24.1	24.8	15.4
	(KW)	3.4	3.0	2.5	4.2	6.5	4.8	3.6
	ACH50	11.8	13.7	12.9	11.4	13.2	12.6	16.8
	(ANOVA)	1.7	1.0	0.9	0.4	1.7	1.1	2.7
	Lysozyme	54.0	45.7	55.1	28.5	58.4	49.1	57.0
	(KW)	10.0	11.0	6.4	6.4	8.8	6.7	11.4
anti oxidant parameters- liver	SOD	563^{ab}	1373^b	212^a	417^{ab}	527^{ab}	1220^{ab}	818^{ab}
	(KW)	163	280	92	108	111	341	462
	Catalase	75.2	110.6	128.9	73.2	130.3	128.4	145.8
	(KW)	7.6	9.8	13.4	7.7	27.5	17.7	24.1
	GPx	51.4	47.0	44.0	56.4	80.0	48.0	51.6
	(ANOVA)	9.8	6.1	7.6	7.7	15.7	9.4	7.6
	GSht	7.5^a	9.6^{ab}	9.4^{ab}	7.3^a	13.1^{ab}	12.8^{ab}	17.2^b
	(KW)	0.9	1.2	1.6	1.0	2.8	2.1	2.4
anti oxidant parameters- gill	SOD	341.0	731.3	707.4	425.2	731.5	416.6	620.5
	(KW)	73.2	221.5	131.6	93.1	235.0	59.0	91.5
	Catalase	28.0	48.3	33.8	42.3	50.4	22.5	24.5
	(KW)	4.5	7.7	4.7	7.3	10.8	2.0	3.0
	GPx	50.9^{ab}	76.0^a	57.4^{ab}	64.0^{ab}	77.5^a	49.6^{ab}	42.1^b
	(ANOVA)	5.0	9.8	7.8	5.5	8.4	6.7	6.2
	GSht	15.9^a	27.4^b	21.2^{ab}	19.3^{ab}	26.8^b	18.6^{ab}	16.9^a
	(KW)	1.2	2.8	2.5	1.9	1.3	2.5	0.8

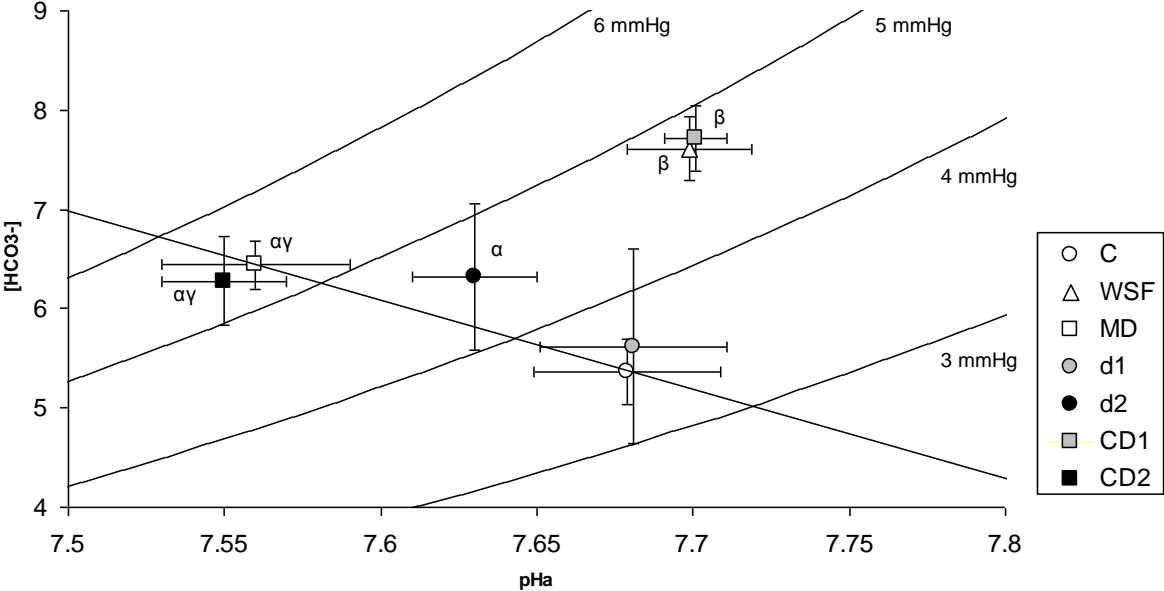
C: Control group, WSF: Water Soluble Fraction of oil, MD: Mechanical Dispersion, d1 & d2: dispersants, CD1 & CD2: Chemically Dispersed oil. Mean values \pm sem. n=10. Letters indicate significant differences between groups (ANOVA or Kruskal Wallis -KW- tests)

Table 4 : Contamination level, stress and metabolism, blood homeostasis, immune function and anti-oxidant systems after 15 days of recovery.

		C	WSF	MD	d1	d2	CD1	CD2
biliary fluo.	343:383	3.88	2.48	3.08	4.11	2.65	3.01	3.44
	(ANOVA)	0.54	0.21	0.19	0.73	0.16	0.29	0.27
	380:430	2.16	1.92	2.77	2.61	1.44	1.96	2.37
	(ANOVA)	0.22	0.17	0.22	0.46	0.17	0.21	0.26
stress indicators	Cortisol	7.80	19.78	12.39	19.71	24.86	26.60	20.50
	(KW)	2.49	4.74	4.26	5.63	6.42	7.33	5.91
	Glucose	32.90	36.91	33.56	36.00	33.50	35.10	32.60
	(KW)	1.57	2.15	1.95	2.80	1.59	1.47	1.44
	Lactate	28.85	58.14	22.43	42.88	30.89	42.95	27.98
	(KW)	6.03	14.29	5.13	11.13	5.82	13.08	7.74
hydromineral balance	Osm	297.1	302.0	301.0	300.3	298.3	297.4	302.2
	(ANOVA)	1.0	2.3	2.1	3.5	3.0	2.2	3.5
	[Na+]	152.6	147.0	149.0	148.8	149.3	148.0	147.8
	(ANOVA)	3.2	2.1	2.4	2.0	2.0	2.4	3.1
	[Cl-]	108.5	106.3	104.0	105.8	104.2	102.3	105.7
	(ANOVA)	1.5	1.4	2.9	1.2	1.3	1.5	1.1
acid-base balance	pH	7.62	7.61	7.63	7.59	7.61	7.63	7.65
	(ANOVA)	0.04	0.03	0.02	0.03	0.04	0.04	0.02
	[HCO3-]	5.17	5.88	6.18	6.34	5.33	5.34	5.47
	(ANOVA)	0.61	0.29	0.43	0.41	0.49	0.27	0.30
	pCO2	4.45	4.51	4.32	5.09	3.84	4.05	3.85
	(ANOVA)	0.54	0.31	0.48	0.41	0.50	0.49	0.39
haematology	leucocytes	110.9^a	90.9^{ab}	61.5^b	92.2^{ab}	75.6^{ab}	65.9^b	55.0^b
	(ANOVA)	18.0	7.8	5.3	10.7	6.1	15.0	5.3
	granulocytes	20.2	19.3	29.4	15.5	10.7	26.6	24.6
	(ANOVA)	5.6	3.7	5.7	2.5	2.3	7.0	3.7
	lymphocytes	90.7^a	71.6^{abc}	32.1^{bc}	76.6^{ab}	65.0^{abc}	39.4^{abc}	30.4^c
(KW)	15.7	9.2	5.8	9.7	6.2	11.1	5.5	
	leu. mortality	1.02	1.08	1.32	1.03	0.98	1.02	1.02
	(KW)	0.32	0.12	0.22	0.12	0.09	0.11	0.22
immune parameters	phagocytosis	25.3	19.4	18.2	23.1	28.7	27.7	21.5
	(KW)	2.3	3.0	2.9	2.3	2.9	4.3	3.7
	ACH50	18.8	18.1	19.0	19.5	15.2	20.9	19.4
	(KW)	1.8	0.8	1.9	2.0	1.6	2.1	1.7
	Lysozyme	56.1	60.7	68.2	72.8	63.8	96.6	73.2
	(KW)	10.7	9.1	7.7	11.3	10.5	12.6	11.7
anti oxidant parameters- liver	SOD	162	799	67	716	380	948	739
	(KW)	53	556	37	475	323	420	263
	Catalase	145.3^a	94.7^{ab}	85.2^{ab}	149.5^{ab}	129.7^{ab}	73.5^b	122.1^{ab}
	(KW)	16.8	20.2	13.5	67.9	49.7	12.9	25.4
	GPx	153.4	80.7	69.3	120.4	172.9	62.7	76.4
(ANOVA)	39.4	18.5	12.2	56.9	80.9	8.8	7.3	
	GSHt	20.0	15.5	12.2	21.0	18.7	12.5	20.5
	(KW)	2.8	2.9	1.3	8.2	6.1	1.3	2.7
anti oxidant parameters- gill	SOD	1271^{ab}	1377^{ab}	2522^b	1226^{ab}	1218^{ab}	780^a	912^{ab}
	(KW)	270	377	452	286	217	100	221
	Catalase	38.7	36.8	26.7	31.6	28.3	31.6	37.9
	(KW)	4.0	6.0	6.2	6.5	3.7	7.2	5.8
	GPx	98.2	87.8	100.9	97.4	72.5	87.0	74.0
(ANOVA)	12.8	7.9	6.4	9.1	5.7	8.5	7.5	
	GSHt	34.6	29.8	30.8	33.1	30.9	31.8	27.3
	(ANOVA)	3.0	2.2	2.6	4.0	2.1	3.3	2.6

C: Control group, WSF: Water Soluble Fraction of oil, MD: Mechanical Dispersion, d1 & d2: dispersants, CD1 & CD2: Chemically Dispersed oil. Mean values \pm sem. n=10. Letters indicate significant differences between groups (ANOVA or Kruskal Wallis -KW- tests)

Fig. 1: Acid base equilibrium after contamination.



Statistical difference with control group; α : pH difference; β : $[HCO_3^-]$ difference; γ : pCO_2 difference.

Fig. 2:

Principal component analysis after 48 h of contamination; projection of the quantitative variables onto the correlation circles

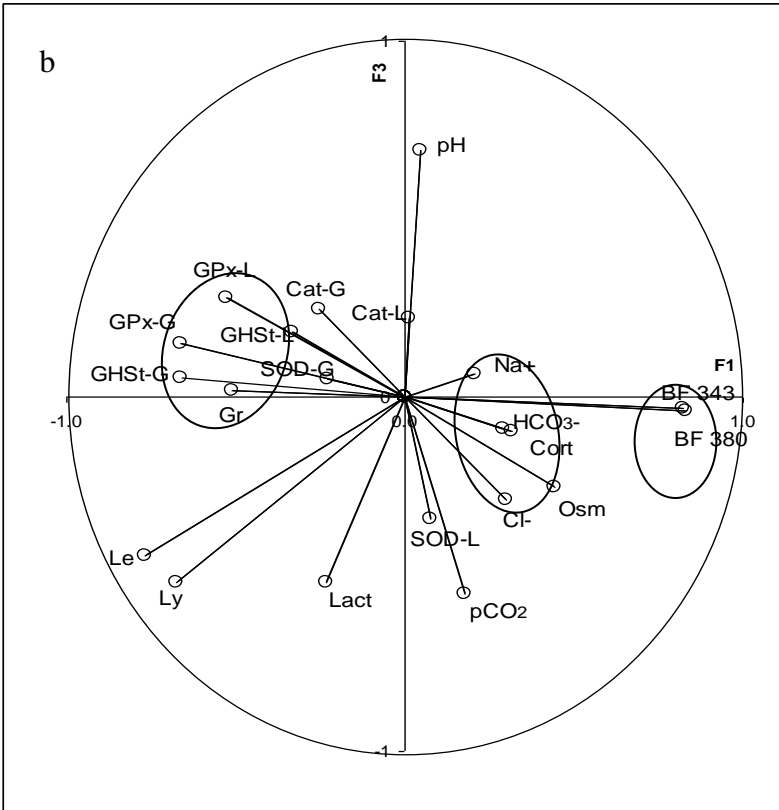
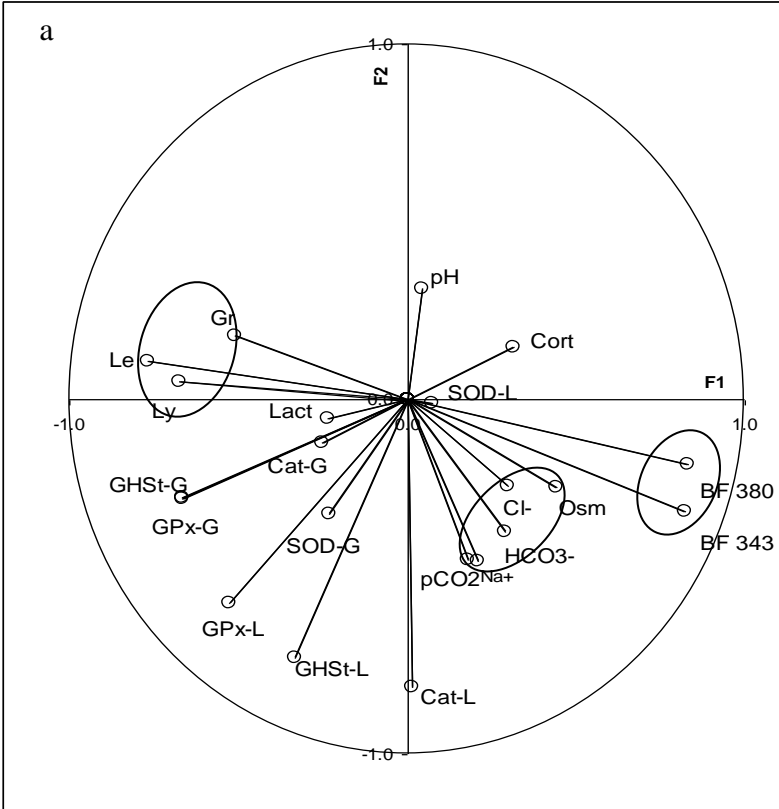


Fig. 3: Principal component analysis; distribution of the individuals in the factorial plan (F1*F2) after 48 h of contamination and 15 days of recovery

