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Responses of the European flounder (*Platichthys flesus*) to a mixture of PAHs and PCBs in experimental conditions

Célie Dupuy[1, 2, *], Claire Galland[1], Alain Devaux[3, 4], Sylvie Bony[3, 4], Véronique Loizeau[5], Morgane Danion[6], Vianney Pichereau[1], Michel Fournier[2], Jean Laroche[1]

[1] UMR 6539 CNRS/UBO/IRD/Ifremer, Laboratoire des Sciences de l'Environnement Marin LEMAR, Institut Universitaire Européen de la Mer, Université Européenne de Bretagne, Université de Bretagne occidentale, Plouzané, 29280, France
[2] Institut National de la Recherche Scientifique-Institut Armand-Frappier (INRS-IAF), 31 Boulevard des Prairies, Laval, Québec, H7V 1B7, Canada
[3] Université de Lyon, UMR 5023 LEHNA CNRS, Villeurbanne, F-69100, France
[4] INRA, USC 1369 IGH, ENTPE, F-69518, Vaulx-en-Velin, France
[5] Unité Biogéochimie et Ecotoxicologie, IFREMER, Centre de Brest, BP70, Plouzané, 29280, France
[6] Laboratoire Ploufragan-Plouzané, Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail, ANSES, Technopôle Brest-Iroise, Plouzané, 29280, France

*: Corresponding author : Célie Dupuy, email address : cel.e@hotmail.fr

Abstract:

A multibiomarker approach was developed to evaluate the juvenile European flounder responses to a complex mixture of 9 polycyclic aromatic hydrocarbons (PAHs) and 12 polychlorinated biphenyls (PCBs). Exposure was performed through contaminated food pellets displaying: (1) PAH and PCB levels similar to those detected in the heavily polluted Seine estuary, respectively in sediments and in flatfish and (2) ten times these concentrations. Several biomarkers of the immune system (e.g., lysozyme concentration and gene expression of complement component C3 and TNF-receptor), DNA damage (e.g., Comet assay), energetic metabolism (e.g., activity of cytochrome C oxidase), detoxification process (e.g., cytochrome P450 1A1 expression level: CYP1A1; betaine homocysteine methyl transferase expression level: BHMT) were investigated after 14 and 29 days of contamination, followed by a 14-days recovery period. After 29 days of contamination, the detoxification activity (CYP1A1 expression level) was positively correlated with DNA damages; the increase of the BHMT expression level could also be related to the detoxification process. Furthermore, after the recovery period, some biomarkers were still upregulated (i.e., CYP1A1 and BHMT expression levels). The immune system was significantly modulated by the chemical stress at the two concentration levels, and the lysozyme appeared to be the most sensitive marker of the mixture impact.

Keywords: Mixture of contaminants ; Fish ; Biomarker ; Immunotoxicity ; Detoxification process ; DNA damage ; PAHs ; PCBs

1 INTRODUCTION

Aquatic environments can be affected by complex mixtures of chemicals, including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), heavy metals, pesticides and endocrine disrupting chemicals. The safety levels of these contaminants are based on single substance exposure studies; however when present in a mixture, a contaminant can be more or less toxic because of possible chemical interactions between toxicants, commonly called the "cocktail effect" (Celander 2011). Thus in different mixtures, contaminants can produce synergistic or antagonistic effects on the exposed organisms.

The European flounder, *Platichthys flesus* (Linnaeus, 1758), is considered as a pertinent sentinel species for water quality monitoring in coastal marine systems (SGIMC, 2011); thus, numerous studies have assessed the biological effects of contaminants on natural populations of *P. flesus* (*e.g.*, Evrard et al. 2010a; Vethaak et al. 2011). In field studies, contaminant load and responses from the subcellular (*i.e.*, molecular responses, such as the modulation of biotransformation enzyme activity, DNA damages, etc.) to the organism level (*i.e.*, growth, disease occurence, etc.) are nowadays frequently assessed in order to explore possible cause-and-effect relationships between chemical stress and biological responses; this integrative approach on the flounder responses in estuaries is usually efficient to diagnose the general level of chemical stress (Williams et al. 2011; Laroche et al. 2013). However, complementary studies must be conducted in laboratory to better explore the natural variability (*i.e.,* noise) *vs* the contaminant-induced stress (*i.e.,* signal) for numerous biomarkers in the context of pollutant mixtures.

The present study is exploring 1) the baseline of several biomarkers involved in the immunity, metabolic rate and DNA damage, and 2) their responses for flounders exposed to a mixture of PAHs and PCBs in experimental conditions. A cocktail of 9 PAHs and 12 PCBs

commonly observed in the field was prepared. Two exposures were performed through contaminated food pellets displaying : (1) PAH and PCB levels similar to those detected in the heavily polluted Seine estuary, respectively in sediments (Cachot et al. 2006) and in flatfish (Loizeau and Abarnou 1995; Abarnou and Duchemin 2008; Gilliers et al. 2004) and (2) ten times these concentrations.

Flounders were contaminated by the pellets during 29 days, then fed with uncontaminated food for a 14 days recovery period. Immune markers were analyzed during this experiment, considering particularly the lysozyme activity and the gene expression level of complement unit 3 (C3) and Tumor Necrosis Factor Receptor (TNF-R). Contaminants may modulate the immune system by immunosuppression, autoimmunity reactions or hypersensitivity responses (Zelikoff 1998). Immune parameters can be modulated by many stressors and thus are not considered as specific markers (van der Oost et al. 2003); however, immunotoxicologic approaches appear very pertinent in ecotoxicology because alterations of the immune functions may conduct to an increased fish susceptibility to parasites and thus to a lower fitness (e.g., Dautremepuits et al. 2004; Danion et al. 2012).

Lysozyme is an enzyme of the innate immune system, playing a role in the protection against microbial invasion (Lie et al. 1989). Lysozymes show lytic activity against gram-positive and gram-negative bacteria and play a role in the activation of complement sytem and phagocytes (Saurabh and Sahoo 2008). Lysozyme level is sensitive to environmental contaminants and is measured on the serum (easy and inexpensive analysis); thus, it could be a convenient parameter for monitoring the impact of pollutants (Bols et al. 2001).

Furthermore, the expression level of two genes (C3 and TNF-R) was also measured in the flounder liver. Complement C3 is a central protein involved in both classical and alternate pathways of complement (Rehana and Kini 2008). The complement system is involved in modulation of phagocytosis, lysis of cells and the inflammatory response (Ye et al. 2011;

98 Boshra et al. 2006). A modulation of C3 mRNA expression was observed in fish after a long-
99 term exposure or between fish living in pristine vs polluted habitats; thus several studies
100 observed an induction of C3 mRNA by the pollutants (Holth et al. 2010; Straub et al. 2004).
101 On the other hand, Williams et al (2003) showed a down-regulation of C3 mRNA in
102 European flounders living in a contaminated estuary.

103 Tumor necrosis factor (TNF) and its cellular receptor (TNF-R) are proteins implicated
104 in cellular signaling pathways involving inflammation, apoptosis, necrosis, lymphocyte
105 homeostasis (Smith et al. 1994; Wiens and Glenney 2011). TNF-alpha is a TNF that can also
106 modulate the biotransformation process by the down regulation of Cytochrome P450 activites
107 in fish (Reynaud et al. 2005). Moreover, Bado-Nilles et al. (2011) observed an up-regulation
108 of TNF-alpha on European seabass, *Dicentrarchus labrax*, after 7 days of exposure with light
109 cycle oil. No sequence of TNF-alpha being known for the European flounder, *P. flesus*,
110 therefore we decided to study the expression of the corresponding cell-surface receptors
111 (TNF-R).

112 The molecular responses to chemical stress were also analyzed in flounder livers,
113 considering the expression level of two genes: the betaine homocysteine S-methyltransferase
114 (BHMT) and the Cytochrome P450 1A1 (CYP1A1). BHMT is an enzyme implicated in the
115 regeneration of methionine by remethylation of homocysteine. Induction of BHMT was
116 underlined in several studies on the flounder responses to contaminants in experimental
117 context and in the field which suggested that BHMT could be involved in detoxification
118 process (Evrard et al. 2010a; Marchand et al. 2006). CYP1A1 has an important function in the
119 biotransformation of many xenobiotics (Sarasquete and Segner 2000). Induction of flounder
120 mRNA CYP1A1 by the pollutants was also demonstrated in laboratory and in the field
121 (Eggens et al. 1996; Evrard et al. 2010a). Moreover, several studies suggested that the

122 immunotoxic effects of contaminant could be induced by the upregulation of CYP1A1 (e.g.,
123 Carlson et al. 2004).

124 The flounder cellular responses to chemical stress were also considered in this study
125 through the assessment of DNA damage in erythrocytes, using the Comet assay. This assay
126 reveals a large array of DNA damage such as DNA single and double-strand breaks, alkali
127 labile and incomplete repaired sites at the cellular level. Several studies have underlined the
128 interest of the Comet assay to assess the genotoxic effects of contaminants such as PAHs and
129 PCBs in fish tissues (e.g., Devaux et al. 1998; Flammarion et al. 2002; Costa et al. 2008).

Lastly, the cytochrome C oxidase (CCO) activity was measured in fish muscle. CCO is the terminal enzyme of electron transport chain of the mitochondria (Complex IV); this aerobic enzyme may constitute an excellent proxy of the fish metabolic capacity (Cohen et al. 2005; Pelletier et al. 1994).

The major objective of the present study is to integrate the molecular, cellular and physiological responses of the flounder to the food contamination with a mixture of PAHs and PCBs, during a one month period, followed by a recovery period where the reversibility of the different marker responses will be explored.

130 ## 2 MATERIALS AND METHODS

131 ### *Experimental design*

132 Juveniles of *P. flesus* (cohort 0+, average weight: 25.2±8g and average total length:
133 11.9±1.5cm) were purchased in a farm (Fishlab, Denmark) in october 2010, then reared in our
134 laboratory in experimental tanks, the water being directly collected in the Bay of Brest
135 (salinity ≈ 35g/l) and maintained at a temperature between 14 and 17°C. After a 14 days
136 acclimation period to the laboratory conditions, several fish groups were contaminated

137 through food, by a mixture of pollutants (PCBs and PAHs). Levels and profiles of PCBs and
138 PAHs were representative of those detected in sediments and fish of the Seine estuary (C1
139 condition). C2 condition was characterized by a 10 times higher concentration. Mixtures were
140 prepared and pellet contaminant load was measured by the LABOCEA laboratory (public
141 laboratory, Plouzané, France), in order to check the efficiency of the impregnation (Table 1).
142 Six fish groups were considered: a control group (pellets without iso-octane), a control group
143 with the solvent (pellets with iso-octane) and two contaminated groups (C1 and C2) in batch
144 replicates.

145 After the acclimation period, 10 fish were sacrificed as a control (t_0), then 10 fish per
146 group were sacrificed after 14 and 29 days of contamination. After this contamination period,
147 fish were fed with normal commercial pellets and 10 fish per group were sacrificed after a 14
148 days recovery period (*i.e.* 45 days after t_0). As a whole, in this work, 200 fish were sacrificed :
149 10 fish control and 10 fish control with solvent at t_0, then 10 fish control, 10 fish control with
150 solvent and 10 fish in C1 and C2 group in duplicate at each sampling date.

151 One day before t_0, animals were tagged and their standard length (Lsd, *i.e.* until the
152 hypural joint) and total weight were measured to the nearest 0.1cm and 0.1g respectively.
153 Each sampling operation was conducted with the following protocol:

154 (1) The Lsd and total and gutted weight were measured per fish, and a blood sample
155 was collected from the caudal vein with a heparinized syringe. One part of each blood sample
156 was diluted (1/100) in a cryopreservative buffer (Evrard et al. 2010a) for the Comet test and a
157 second part was centrifuged (5 min, 5000 G) to obtain plasma for the lysozyme activity
158 analysis. All samples were frozen in liquid nitrogen.

159 (2) The fish were killed by concussion and fragments of liver and muscle were quickly
160 dissected and frozen in liquid nitrogen, eviscerated fish being stored at -20°C for further PCB
161 analysis.

Visual inspection did not reveal any trace of liver pathology or parasite infection. All experiments were performed according to the guidelines laid down by the French "Ministère de l'Agriculture" and the European Union council directive for the care and use of laboratory animals.

Biometric indices

The Fulton's condition factor (CF) was calculated by the formula:

$CF = 100 \times W / Lsd^3$, where W is the fish gutted weight (g) and Lsd is the standard length (mm) of each fish.

The specific growth rate (SGR) of each fish was estimated using the formula: SGR (% per day) = $100 \times (\ln (W_t) - \ln (W_0)) / (t - t_0)$, where W_0 and W_t are the fish weights at t_0 and t days respectively (Talbot 1993).

Food preparation and PCB analyses in food and fish tissues

Commercial food (Inicio plus, 1.9mm), obtained from Biomar (France), consisted of a formulated feed containing 61% crude protein and 33% crude fat. Spiked food was prepared by batch to avoid deterioration and by slowly adding a solution in iso-octane of known amounts of twelve PCB congeners and 9 PAH, (AccuStandard Inc., New Haven, USA, purity above 98%) to food pellets in order to prepare diets (Table 1). PCBs mixtures were composed of the seven indicator PCB congeners (CB28, 52, 101, 118, 138, 153, 180), and a few others to describe a larger range of chlorination from 3 to 8 chlorine atoms (CB105, 149, 156, 170, 194). The latter compounds were chosen because they can highlight any underlying mechanisms acting on the distribution of organic contaminants in biota, i.e., bioaccumulation or biotransformation. CB153 is not metabolized and is used as a representative of all twelve congeners. PAHs mixtures were composed of 9 PAHs of high molecular weight (three to five

rings) found in European and American estuaries and already used in several studies of contamination with PAHs mixtures (e.g., Arkoosh et al. 2001; Bravo 2005; Bravo et al. 2011).

Two diets were prepared: medium and high PCBs & PAHs mixtures for which targeted concentrations of individual congeners are presented in Table 1. PAHs being metabolized, only the PCBs concentrations were assessed to confirm the fish contamination. Batches of spiked food were stored in amber containers in a cool, dark place throughout their use (circa 1.5 months). Individual PCB congener concentrations were determined in each batch of spiked food (Table 1). Non-spiked food was also analyzed for PCB levels, and concentrations were between <0.05 ng/g dry weight (dw) and 3.0 ng/g dw (mean value calculated on 5 replicates) depending on the congener. In addition to congeners included to spike the contaminated food, CB 31 (not detected; nd), CB 77 (nd), CB110 (0. 75 ng/g dw), CB132 (nd), CB128 (nd), CB187 (nd) non-added congeners and p,p'-DDE (3.50 ng/g dw) were measured.

The PCB analysis in eviscerated fish was performed according to the protocol described by Bodiguel et al. (2009). One gram of freeze-dried eviscerated fish was spiked with recovery standards (CB30, CB198, CB209) and was extracted in a hot Soxhlet extraction apparatus (Soxtec) over 1.5 h with a hexane:acetone mixture (80:20). The amount of extracted fat was determined by gravimetric analysis. Then, two successive cleanups were performed on the extractible material. First, lipids and co-extractible material were destroyed by adding concentrated sulphuric acid. The cleaned extract was then purified by adsorption chromatography on a Florisil column (16% MgO + 84% SiO2, activated for 1.5 h at 500◦C, deactivated with 3% of demineralised water) and eluted with pentane. Finally, PCB congeners were analyzed by gas chromatography with an electron capture detector, on a HP 5890 series II equipped with a CP-Sil19 (60 m length, internal diameter 0.25 mm, and 0.15 µm phase film thickness) and HT8 (50x0,22x0,25) capillary columns following optimized conditions

described by Jaouen-Madoulet et al. (2000). The system was calibrated within quite a large range using a six-point calibration curve to define the linearity range of our detector (ECD) for all contaminants. The relative precision of the method was checked for this type of sample by analyzing five aliquots of a homogeneous tissue preparation of fish muscle. The results showed standard deviations of less than 10% for all congeners, indicating that the method had a satisfactory reproducibility. During analysis of the real samples, analytical blanks were systematically measured for every ten samples. The blank concentrations were always less than the concentrations of the lowest standards of all congeners.

Finally, 5 replicates of a reference material, BCR-SRM1588 (Cod Liver Oil) were analyzed to determine the accuracy and precision of the method. PCB recoveries varied between 74% and 125%. Furthermore, the RSD values ranged from 4% to 19%, with a mean of 10% for all PCBs. All of these results were in agreement with certified reference values and published data (Schantz et al. 1993). The BE-Ifremer unit takes part in Quality Assurance of Information for Marine Environmental Monitoring in Europe (QUASIMEME); inter-comparison exercises for PCBs in biota and Z-scores were satisfactory (i.e., between −2 and +2).

Comet assay

In this study, the Comet assay was carried out on flounder erythrocytes according to the procedure described by Singh et al. (1988) with slight modifications detailed in Evrard *et al.* (2010a). After cell lysis and electrophoresis, DNA was stained with 0.05 mM ethidium bromide and scored using an Axioskop epi-fluorescence microscope (Zeiss™) and the Comet assay IV image analysis system (Perceptive Instruments Ltd., Haverhill, UK). Randomly selected cells from two replicated slides (50 cells per slide) were analysed. The percentage of

DNA in the Comet tail (median % tail intensity) was chosen as the most reliable and meaningful Comet measurement (Jha 2008).

Lysozyme

The assessment of plasma lysozyme level was carried out by the turbidimetric method of Parry et al. (1965). A suspension of 110 µL *Micrococcus lysodeikticus* (10 µg/ml, 0.05 M sodium phosphate buffer, pH 6.4) was mixed with 40 µL of serum in a 96-well plate. The reaction was carried out at room temperature and read at 450 nm at 1 min intervals for 20 min, homogenizing before each run. On each 96-well plate, the measure was also carried out on a known lysozyme concentration standard (0 to 0.75 µg/mL) to calculate the concentration of lysozyme in each plasma sample. Protein concentrations in plasma sample were determined by the method of Bradford using bovine serum albumin as a standard (BIO-RAD Laboratories, USA).

CCO (cytochrome C oxidase)

The CCO activity was measured at 22°C by a microplate spectrophotometric assay in muscle S9 fraction (Smith and Conrad 1956; Théron et al. 2000). Absorbance changes were monitored at 550 nm during 8 min. Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard (BIO-RAD Laboratories, USA).

Gene expression

Total RNA was isolated using Trizol reagent (Applied Biosystems™) at a concentration of 1 mL/50 mg of tissue. Each liver was homogenized in Trizol using a Precellys-24® ceramic bead-based homogenizer (Bertin Technologies™). Samples were treated with RTS DNAse Kit (MO BIO) to prevent DNA contamination. The purity and

255 concentration of samples were determined using a NanoDrop 8000® spectrometer (Thermo
256 Scientific™). Purity was assessed using the ratios A260/A230 and A260/A280. RNA quality
257 was assessed by capillary electrophoresis using the Agilent RNA 6000® Nano Assay Kit on
258 the Agilent 2100® bioanalyser (Agilent Technologies™) or by migration on agarose gel
259 (1.5%). Reverse transcription PCR (RT-PCR) was carried out using 1 µg total RNA from
260 each sample with RevertAid H minus First Strand, cDNA Synthesis Kit® (Fermentas™). A
261 RT-PCR was carried out using a RNA mixture of samples coming from each experimental
262 condition. This mixture is a control used on each run of real-time quantitative PCR (qPCR).

263 The mRNA expression level was explored for the following genes: BHMT, C3, TNF-
264 R and CYP1A1. Two housekeeping genes were tested: alpha-tubulin and 18S. No significant
265 differences of CP (crossing point threshold) were observed for alpha-tubulin between the
266 control and the contaminated fishes; furthermore Alpha-tubulin showed the lowest variation
267 coefficient, and thus was retained as the housekeeping gene in our analysis. Primers of
268 CYP1A1, Alpha-tubulin, 18S and BHMT were obtained in the literature (Table 2). Primers of
269 C3 and TNF-R were designed considering the sequences available on Genbank and using
270 Primer 3 software (Rozen and Skaletsky 1999).

271 The real-time qPCR amplifications were carried out in triplicate with the Absolute
272 QPCR SYBR Green ROX Mix® (Thermo Scientific™). The final volume of reaction was of
273 25 µL with 1 µL of cDNA (1/40 dilution), 12.5 µL of ABsolute QPCR SYBR Green Mix®
274 (Thermo Scientific™), 1.75 µL of each primer (1 µM) and 8 µL of water. A negative control
275 was carried out (each total RNA sample with DNAse treatment). Each run included cDNA of
276 sample, cDNA of mixture used as control, blank controls (water) analyzed for each primer
277 pair and a cDNA mixture analyzed for the housekeeping gene and used to normalize the
278 plates (and take into account variations between plates). The qPCR was carried out using a
279 7300 Real-Time PCR System® (Applied Biosystems™) with the following qPCR thermal

cycling program : 50°C for 2 min, 95°C for 10 min, then 40 cycles at 95°C for 15 s followed by 1 min at 60°C. A melt curve was carried out to confirm the specificity of the reaction with the following program : 95°C for 15s, 60°C for 30s and an increase of temperature by 0.5°C each 15s to 90°C. PCR efficiency (*E*) was determined for each primer pair by determining the slopes of standard curves obtained from serial dilution analysis of cDNA. Relative expression of the target gene was calculated with the Pfaffl formula (2001) using Alpha-tubulin as the reference gene :

$$Ratio = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}}$$

The ratio of a target gene is expressed in a sample versus a control in comparison to a reference gene, where, Etarget is the qPCR efficiency of a target gene transcript; Eref is the qPCR efficiency of the reference gene transcript; ΔCPtarget is the CP (crossing point threshold) deviation of control – sample of the target gene transcript; ΔCPref = CP deviation of control – sample of reference gene transcript.

Statistical analysis

Statistic tests and box plots were carried out using Statistica 10.0 (StatSoft™). Data were not normally distributed, so the Kruskal-Wallis (KW) test was used for inter-condition comparisons. When KW test was significant ($p \leq 0.05$), it was followed by a post-hoc test: a multiple bilateral comparison of mean ranks. No significant difference was detected over the whole markers (KW test: $p > 0.05$) between control group and control group with solvent, between the two groups of C1 and between the two groups of C2, thus the analysis were conducted pooling the two groups of control, the two groups of C1 and of C2.

Principal Component Analysis (PCA) was used to develop an integrative approach on the fish responses to contaminants after 14 and 29 days of exposure, considering the three fish

groups; control / exposed to C1 / exposed to C2. The following variables were considered in the PCA: Lsd, SGR, DNA damage, lysozyme concentration, CCO and gene expression; all data being standardized (zero mean, unit variance) before the analysis. This analysis produced 1) a correlation circle allowing to explore the relationships between the quantitative variables, and 2) the fish distribution on the main factorial plan.

3 RESULTS

Chemical analyses of pellets and eviscerated fish, and biometric indices

Chemical analyses of pellets indicated that the concentrations of PCBs/PAHs were close to the intended concentrations (Table 1), for both the moderate and the high load of contaminants (C1 and C2).

After the 29 days contamination period, the concentration of PCBs in eviscerated fish was close to the expected concentration, C2 concentration being tenfold higher than C1 (Fig 1). Furthermore, even after the 14 days restoration period, the levels of PCBs in the eviscerated fish remained high; although a moderate decrease of the contaminant load was detected in the C1 and C2 fish groups, this trend being lower in the C1 fish group (Fig 1).

No significant difference of CF, Lsd was detected between the different fish groups (control/exposed to C1/exposed to C2) after 14, 29 or 43 days of experimentation.

Comet assay

After 14 days of contamination, no significant differences appeared between the blood cells DNA damages in control *vs* exposed fish at both concentrations (Fig 2). The increase in DNA damage became significant in C2 group after 29 days of contamination ($p < 0.01$). After the 14 days restoration period, the level of DNA damage was not significantly different

between fish groups and remained low, close to the value observed at the beginning of the experiment (Fig 2). It has to be noticed that for the C2 group after 29 days of contamination, a significant Pearson's correlation ($r = 0.964$, $p < 0.05$) was detected between the level of DNA damage and the mRNA level of CYP1A1 (data not shown).

Lysozyme

A significant decrease of the lysozyme activity was detected after 14 days of contamination for the C1 exposed fish compared to other groups (Fig 3, $p < 0.01$). After 29 days of contamination, the lysozyme activity was significantly reduced for both C1 and C2 groups compared to the control (Fig 3, $p < 0.001$). The levels of lysozyme activity were similar in the three fish groups after the restoration period and not significantly different from levels observed before the contamination.

CCO (cytochrome C Oxidase)

No significant difference was detected for the CCO activities among the fish groups, along the whole experiment (Fig 4); however the highest and the lowest CCO activities were detected for the C2 fish group after respectively 14 and 29 days of contamination.

Gene expression

The BHMT and CYP1A1 expressions were up-regulated for the C2 fishes compared to the other groups after 29 days of contamination ($p < 0.01$); this up-regulation being maintained after the restoration period (Fig 5, respectively $p < 0.01$ and $p < 0.05$). The C3 gene expression was up-regulated for the C1 and C2 groups after 29 days of contamination ($p < 0.01$ and $p<0.001$); the expression levels of this gene were not significantly different between the fish groups after the restoration (Fig 5). The TNF-R gene expression was up-

regulated in the C2 fishes compared to other groups, after the 29 days contamination period (Fig 5, $p < 0.01$).

PCA

Two PCAs were carried out on the fish responses after 14 and 29 days of contamination. After the 14 days contamination period, the first and second principal components (axis 1 & axis 2) accounted for respectively 38.34% and 16.50% of the total variance over the whole data set (Fig. 6A). On the first axis of the correlation circle, an opposition was observed between the left side of the diagram mainly characterized by high CCO and lysosyme activities, high BHMT and CYP1A1 expression levels and the right side mainly linked to high fish Lsd and SGR (Fig 6A). The second axis of the correlation circle was mainly linked to DNA damage (Fig 6A). The distribution of the individuals on the main factorial plan (axis 1 & 2) showed a mixture of individuals coming from the two groups: control and C1 exposed fish (Fig 6B); the C2 exposed group being mainly localized in the lower part of the diagram and thus showing higher DNA damages. The inter-individual variability in the fish responses of the C2 group was higher to those observed for the control and C1 groups (Fig 6B).

After the 29 days contamination period, the first and second principal components (axis 1 & 2) accounted for respectively 40.36% and 19.42% of the total variance over the whole data set (Fig. 6C). On the first axis of the correlation circle, an opposition was observed between the left side of the diagram mainly characterized by high DNA damage and high expression level of TNF-R, CYP1A1, C3 and the right side of the plan mainly linked to high lysozyme and CCO activities (Fig. 6C). An inverse correlation was detected between the expression level of BHMT in the upper part of the diagram, and the specific growth rate in the lower part of the plan (Fig 6C). The distribution of individuals after 29 days of contamination

showed that the three fish groups (control, C1 and C2) were clearly separated along the first axis (Fig 6D):

- on the right part of the plan, the control fish showed high CCO and lysozyme activities, reduced DNA damages and limited expression levels for TNF-R, CYP1A1, C3 and BHMT;
- on the left part of the plan, the C2 fish displayed an inverse trend, *i.e.*, limited CCO and lysozyme activities, high DNA damages and high expression levels for TNF-R, CYP1A1, C3 and BHMT;
- on the plan center, the C1 fish showed intermediate responses between the control group and the C2 group.

The inter-individual variability in the fish responses of the C2 group after 29 days of contamination was again higher than those observed for the control and C1 groups (Fig 6D).

4 DISCUSSION

Contamination by food and fish chemical load

The fish chemical load clearly showed that all flounders exposed to pollutants, were efficiently contaminated by the food. Chemical analyses underlined a low interindividual variability in the fish PCBs concentrations in each condition. Furthermore, after a 29 days contamination period, the ratio of PCBs loads in the group exposed to the high concentration (C2) / PCB loads in the group submitted to the moderate concentration (C1) was close to 10 (*i.e.* the C2/C1 ratio in the pellets); this result suggests that in the experimental conditions, the fish PCBs bioaccumulation increased linearly with the level of food contamination. After the 14 days recovery period, fish PCBs concentrations remained high for both C1 and C2 concentrations.

394 The cocktail of pollutants C1 was designed to reflect the general level of PAHs and
395 PCBs observed in respectively sediment and biota for heavily polluted systems (Cachot et al.
396 2006; Abarnou and Duchemin 2008). The experimental levels of contamination used in this
397 study allow to explore the complex effects of a cocktail of organic pollutants on detoxification
398 process, DNA damages, immune and metabolic responses, in environmentally realistic
399 concentration and profile. However, the contaminated estuaries showed diversified cocktails
400 of pollutants including organic and metallic compounds, with different modes of toxic action,
401 and thus leading to very complex responses of the organisms in the field.

402 ***Detoxification-biotransformation and genotoxicity***

403 CYP1A1 is implicated in the metabolization of PAHs and of other planar organic
404 pollutants, increasing their hydrosolubility and facilitating further biliary and urinary
405 excretion (van der Oost et al. 2003). Thus, CYP1A1 induction has been used since the 80's as
406 a biomarker of exposure to such environmental compounds in aquatic ecosystems (Payne and
407 Fancey 1982; Stegeman et al. 1987; Monod et al. 1988). However, it is well known that
408 CYP1A1 can contribute to the metabolic activation of PAHs in aquatic organisms, leading to
409 reactive intermediates and the formation of mutagens and carcinogens (Varanasi et al. 1987).
410 In European flounder, an up-regulation of CYP1A1 has been observed both in laboratory and
411 in the field after PCBs and/or PAHs exposure (Eggens et al. 1996; Lewis et al. 2006; Evrard
412 et al. 2010a).

413 In the present study, the PCBs-PAHs cocktail induced an up-regulation of the
414 CYP1A1 expression for the higher concentrated mixture, after a 29 days contamination
415 period. This up-regulation was maintained after the 14 days recovery period and was probably
416 linked to the presence in fish tissues of compounds displaying low biotransformation rates,
417 probably PCBs as suggested by the chemical analysis; thus the PCBs concentrations remained

418 high after the 14 days recovery period, whereas the PAHs were probably quickly metabolized
419 (PAH metabolites have not been measured because of the small-sized gall-bladder).

420 Similarly, the expression of BHMT was also up-regulated after 29 days of
421 contamination with the higher dose. This enzyme, catalyzing the methylation of homocysteine
422 to methionine, is probably involved in phase II detoxification (Marchand et al. 2006; Evrard
423 et al. 2010b; Williams et al. 2008). This up-regulation was maintained after the 14 days
424 recovery period. The convergent results between CYP1A1 and BHMT expressions along the
425 contamination and recovery periods might confirm the implication of BHMT in detoxification
426 processes. Along this experience, proteomic analyses underlined a co-accumulation of
427 BHMT, GST and GPx in European flounders contaminated with this PAHs/PCBs cocktail
428 (unpublished results). It is suggested that BHMT might be involved in a pathway leading to
429 the production of glutathione, allowing the excretion of conjugated xenobitics by GST or anti-
430 oxidative defenses by GPx.

431 DNA damage in flounder erythrocytes assessed in the present study through the
432 Comet assay can be considered as a relevant biomarker of exposure to environmental
433 contaminants as demonstrated in other fish species (Frenzilli et al. 2009; Devaux et al. 1998;
434 Jha 2008). If PAHs metabolites are well-known genotoxicants, PCBs genotoxicity remains
435 more controversial, depending on the PCBs congeners (Belpaeme et al. 1996). In the present
436 study, the increase in DNA damage detected after a 29 days exposure to the highest cocktail
437 concentration followed by the return to its baseline DNA damage level after the restoration
438 period, suggest that PAHs are efficiently biotransformed by CYP1A1 and could mainly be
439 responsible for the measured genotoxicity. This hypothesis is strengthened by the existing
440 correlation between the levels of DNA damage and mRNA CYP1A1 in C2 group after 29
441 days of exposure. A decrease in DNA damage to its basal level after the recovery period

442 indicates that DNA damage was repaired, suggesting that during this period the level of PAH
443 metabolites was probably below the critical threshold value necessary to induce genotoxicity.

444 Genotoxicity observed in flounder exposed to PAHs and PCBs mixture could result
445 from various mechanisms. PAHs exposure can lead to the formation of DNA strand breaks
446 measured by the Comet assay that are the results of incomplete repair of DNA adducts or of
447 alkali-labile sites due to DNA adduct depurination (Speit and Hartmann 1995; Sage and
448 Haseltine 1984). Apart from DNA damage due to highly electrophilic PAHs metabolites,
449 genotoxicity could be due to the generation of reactive oxygen species (ROS) known to take
450 place after PAHs and PCBs exposure, in particular in fish (Schlezinger et al. 2006; Lemaire
451 and Livingstone 1993). Quinone metabolites of PCBs or PAHs can generate ROS by redox
452 cycling finally leading to DNA strand breaks revealed by the Comet assay (Bolton et al. 2000;
453 Ludewig et al. 2000). It is suggested that future exploration on the impacts of mixture of
454 PCBs and PAHs on fish should consider 1) supplementary markers of oxidative stress, 2) to
455 collect bile for the analysis of PAH metabolites, allowing to better explore the relationships
456 between exposure, biotransformation and genotoxicity.

457 In the present study, food contamination level in C1 group did not induce any
458 alteration of CYP1A1 expression or an increase in DNA damage; although similar PAHs and
459 PCBs concentrations led to significant DNA damage in flatfish from the heavily polluted
460 Seine estuary (Akcha et al. 2003). This difference could probably be explained by the rather
461 limited exposure duration time in our experiment (29 days). In the field, juvenile flatfish must
462 stay several months in polluted estuaries before significant biomarker responses could be
463 detected (Evrard et al. 2013). Furthermore, the high complexity of the mixture of pollutants
464 (PAHs, PCBs, heavy metals, pesticides,..) in natural systems could also increase the potential
465 effects of the chemical stress on fish populations.

Immune responses

In the present study, immune biomarkers were clearly modulated by the chemical stress, the decrease of the lysozyme activity being possibly associated to an immunosuppression, whereas the increase of TNF-R and C3 expression levels could indicate an activation of immune system. These results confirm 1) that chemical stress could induce diversified immunomodulatory effects and 2) that a battery of tests is necessary for a better evaluation of the immune system response in polluted environments (Dunier and Siwicki 1993). The inverse trends suggested by lysozyme *vs* TNF-R / C3 results could be explained by the complexity of the immune system; *i.e.*, xenobiotics could lead to alterations that produce apparently contrasting effects: for example, cadmium can elevate lysozyme activity but impair phagocytosis (Bols et al. 2001).

Furthermore, those contrasted trends previously detected must be analyzed cautiously, considering that markers were assessed at both the protein (lysozyme) and mRNA levels (TNF-R and C3). A higher expression of mRNA is not necessarily accompanied by an increase of protein level; *i.e.*, the toxicant which inhibits the activity of protein may increase the transcription of the gene coding for the protein (Nikinmaa and Rytkönen 2011). Moreover, Teles et al (2011) underlined an increase in TNFα mRNA expression in rainbow trout (*Oncorhynchus mykiss*) contaminated with copper, but no change in extracellular TNFα protein concentration was observed, suggesting that translation of TNFα mRNA or secretion of the TNFα protein by the cell is somehow inhibited. In the present experimental context, it is suggested that measures at protein level will be necessary in the future to confirm the induction of TNF-R and C3.

Several authors have evaluated the modulation of TNF system by the measure of TNF-alpha, an extracellular cytokine. But no sequence of TNF-alpha being available for the

European flounder, we chose to study the expression of the corresponding cell-surface receptor (TNF-R), thus exploring an indirect response of TNF system. TNF-R is a receptor of TNF system implicated in apoptosis and cell necrosis (Smith et al. 1994) and playing a role during acute inflammation (Wiens and Glenney 2011). The presence of high contaminant concentrations may be associated with inflammatory response (e.g., Pacheco and Santos 2002; Leaver et al. 2010; Sheir and Handy 2010). In the present study, the induction of TNF-R mRNA could be the result of fish inflammatory response for the higher concentration of pollutants (C2).

Several studies showed an activation of TNF system in fishes exposed to copper (Teles et al. 2011), effluent wastewater (Kerr et al. 2008) and soluble fraction of light cycle oil (Bado-Nilles et al. 2011). Reynaud et al (2005; 2008) observed in carp, *Cyprinus carpio,* an interaction between the immune system and biotransformation, suggesting that the immune system could modulate the biotransformation system; they measured a down regulation in cytochrome P450 content after an injection with TNF-alpha. In the present study, after 29 days of contamination, the up-regulation of TNF-R had not lead to a down-regulation of CYP1A1 but was associated with an up-regulation of CYP1A1. So, if TNF-alpha down regulated the cytochrome P450, this regulation possibly occurred after mRNA expression, or the TNF-R considered in our study like a proxy of TNF-alpha could in fact respond differentially. To better explore the TNF system response, it will be necessary in the future to consider the TNF-alpha specific sequence of the European flounder.

Similar to TNF-R in our experimental conditions, C3 was also up-regulated after 29 days of exposure to the "high" chemical stress; furthermore the C3 up-regulation was also significant for the "moderate" chemical stress. Thus, we suggest that C3 could be a more sensitive biomarker than TNF-R. The complement system is composed of more than 35 soluble plasma proteins and is initiated by one or a combination of four pathways of

activation (Boshra et al. 2006; Amara et al. 2008). C3 is the central protein of complement activation pathways in teleosteans like in human (Boshra et al. 2006; Ye et al. 2011). Complement displays lytic, proinflammatory, chemotactic and opsonic activities, and is associated to nonspecific phagocytic processes (Watts et al. 2001). Some complement components are up-regulated in acute phase responses (Bayne et al. 2001), including the proteins C3 which are upregulated during inflammation (Watts et al. 2001).

Several studies showed an up-regulation of mRNA C3 during an experimental contamination with a complex cocktail of PAHs, alkylphenols and phenol on Atlantic cod, *Gadus morhua* (Holth et al. 2010) or in a field study on winter flounder, *Pseudopleuronectes americanus*, comparing polluted *vs* pristine sites (Straub et al. 2004). In the other hand, Williams et al (2003) observed a down-regulation of C3 in European flounder over polluted sites. Thus, if the C3 and TNF-R mRNA expressions could become pertinent markers in ecotoxicology, more studies will be necessary to better understand the mechanisms of up or down-regulation of these genes by the contaminants.

In the present study, a significant decrease of the lysozyme activity was detected in our contaminated fish groups compared to the control. Fish lysozyme displays lytic activity against both Gram-positive bacteria and Gram-negative bacteria (Saurabh and Sahoo 2008). Exposure to pollutants could modulate lysozyme levels but the nature of the modulation seems complex : (1) serum lysozyme activity was elevated in rainbow trout exposed to cadmium, mercury or zinc (Sanchez-Dardon et al. 1999), (2) plasma lysozyme concentration decreased in flounder contaminated with DDT adducts and PCBs (Skouras et al. 2003), whereas no modification in lysozyme concentration was detected in plasma of sea bass submitted to heavy fuel oil or light cycle oil (Bado-Nilles et al. 2009). These differences of responses could be explained by the type and the concentrations of contaminants; however, in many fish studies, the general decrease of lysozyme in contaminated contexts appears as a

sensitive biomarker (Bols et al. 2001; Saurabh and Sahoo 2008). In our experiment, the measure of lysozyme level appears like a sensitive immune response to contaminants, a signal being detected after only 14 days of exposure in the "moderately" contaminated fish group.

Considering the whole immune responses to contaminants in the present study, we observed after the 14 days recovery period, a restoration of the biological signals towards the initial levels, whatever the considered marker (lysozyme, TNF-R, C3); thus it seems that the contaminants induce a short-time impact on the immune system.

Metabolic activity

In the present study, the estimation of the muscle cytochrome C oxydase activity (CCO) was considered as a potential proxy of the fish metabolic capacity (Cohen et al. 2001). Within each experimental condition (14 and 29 days exposure period, 14 days restoration period) the interindividual variability of the CCO is relatively high in each fish group (control / low contamination / high contamination), thus no significant difference was detected between groups. Again, we suggest that the length of exposure to pollutants in this experiment was too short to induce a significant increase of the energetic metabolism, that was only observed after several months of impregnation for juvenile flounders monitored in polluted systems (Evrard et al. 2013).

Integration of fish responses to chemical stress

After a 14 days contamination period, the factorial analysis underlined the main relationships between the fish groups (control *vs* moderately contaminated *vs* highly contaminated) and the markers. The main part of the variance in PCA was probably not related to the chemical stress, but could be more related to the flounder physiology; in fact, an opposition between two main physiological trends was observed for the flounder, a "high

563 metabolic rate" (high levels for lysozyme, CCO, BHMT and CYP1A1) *vs* a "low metabolic
564 rate" characterized by low levels for the previous markers, and also by high length and
565 specific growth rate. This dichotomy in flounder physiology was first observed in a previous
566 study on juveniles collected in French estuaries: the Seine and the Vilaine (Calvès 2011). A
567 secondary part of the variance in PCA was related to the chemical stress, the higher levels of
568 DNA damage being clearly observed for the highly contaminated fish group.

569 A much contrasted situation was observed by the factorial analysis after a 29 days
570 contamination period. The main part of the variance in PCA was here clearly associated to the
571 chemical stress; *i.e.* the highly contaminated fish group displayed high levels for DNA
572 damage, TNF-R and CYP1A1, these biomarkers being positively correlated. This result
573 confirms 1) the potential relationships between CYP1A1 activity and DNA damages
574 frequently suggested by different authors (e.g., Kleinjans and van Schooten 2002; Roy et al.
575 2003; Wessel et al. 2010) and 2) the link between CYP1A1 activity and immune function in
576 polluted contexts (Reynaud and Deschaux 2006).

577 # 5 CONCLUSIONS

578 The present study underlined an efficient flounder contamination by the food. Thus,
579 the impacts of a mixture of PAHs and PCBs at environmentally comparable concentrations
580 were explored. Particularly after a 29 days contamination period, several biomarkers were
581 clearly affected by the chemical stress. The detoxification activity increased (higher CYP1A1
582 expression level) in the same way to the rise of DNA damages; furthermore, the increase of
583 the BHMT expression level could also be associated to the detoxification process. The
584 responses of the immune sytem to the chemical stress were clearly identified but complex to
585 analyze, considering several modulations in the lysozome activity, and in the expression level
586 of TNF-R and C3.

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905 **Figures**

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908 **Fig. 1.** Measured concentrations of PCBs indicators in eviscerated fish after 29 days of
909 contamination and 43 days of experimentation (29 days of contamination and 14 days of
910 recovery period).

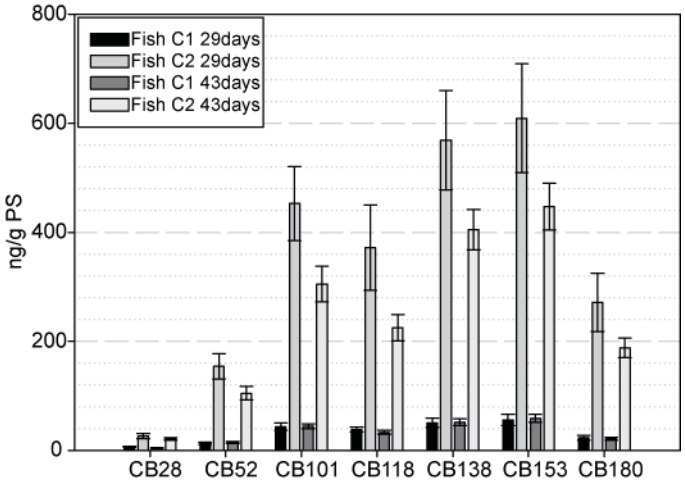

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Fig. 2. Primary DNA damage levels expressed as median tail intensity by the Comet assay during the experiment in control (Ctl) and in contaminated conditions (C1 and C2) after 14, 29 and 43 days. * : $p < 0.05$; ** : $p < 0.01$ and *** : $p < 0.001$.

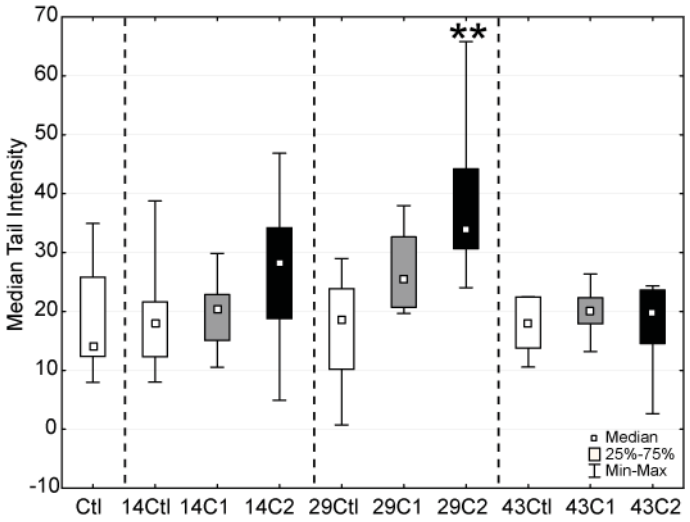


Fig. 3. Lysozyme concentration in plasma during the experiment in control (Ctl) and in contaminated conditions (C1 and C2) after 14, 29 and 43 days. * : $p < 0.05$; ** : $p < 0.01$ and *** : $p < 0.001$.

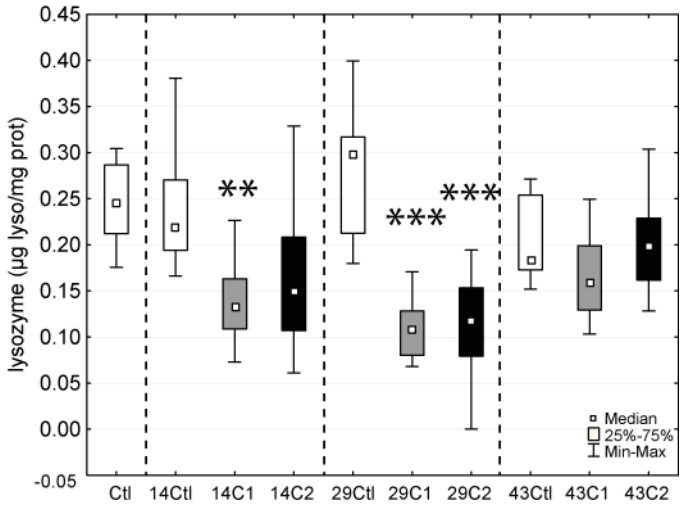


Fig. 4. CCO activity during the experiment in control (Ctl) and in contaminated conditions (C1 and C2) after 14, 29 and 43 days. * : $p < 0.05$; ** : $p < 0.01$ and *** : $p < 0.001$

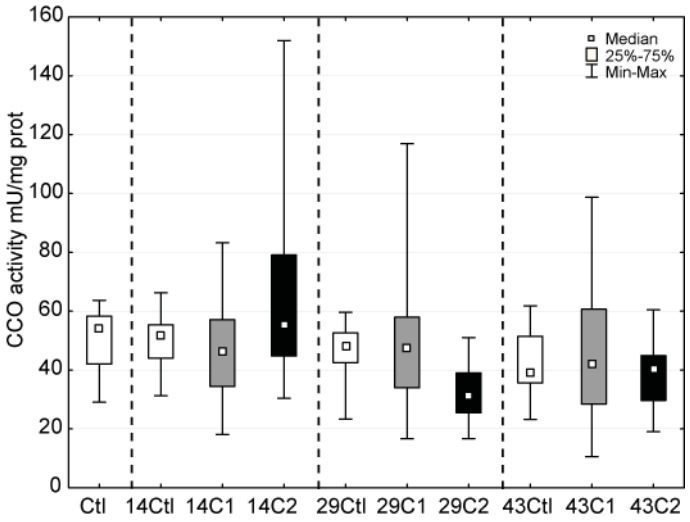


Fig. 5. Expression level of target gene compared to the reference gene (α tubulin) during the experiment in control (Ctl) and in contaminated conditions (C1 and C2) after 14, 29 and 43 days. * : $p < 0.05$; ** : $p < 0.01$ and *** : $p < 0.001$.

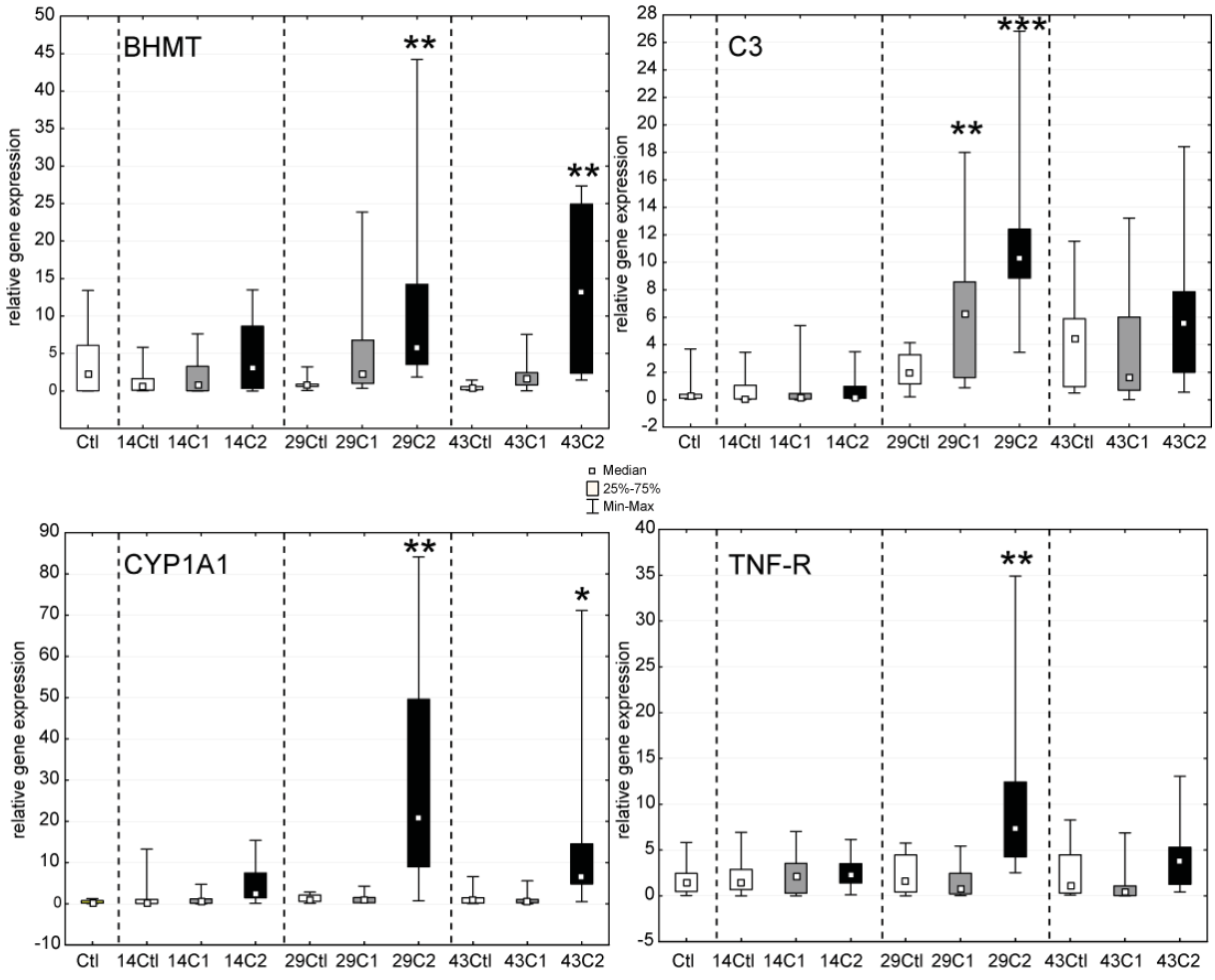
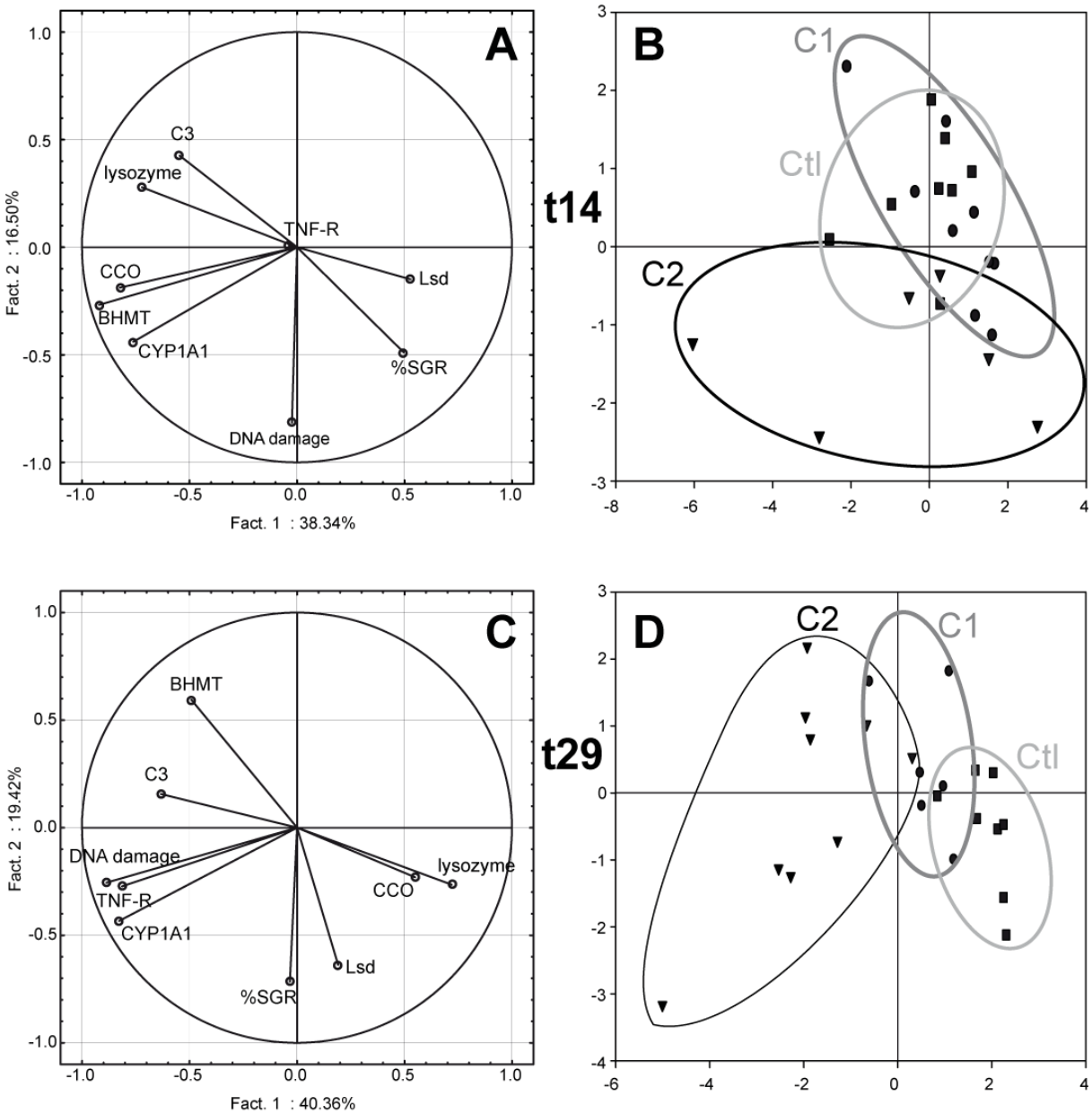


Fig. 6. Principal Component Analysis (PCA): distribution of the variables on the correlation circle after 14 days (A) and 29 days (C) of contamination and distribution of the fishes on the main factorial plan after 14 days (B) and 29 days (D) of contamination, considering the three fish groups (Ctl, C1 and C2).



945 **Tables**

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948 **Table 1. Targeted and measured concentrations (ng/g) of PAHs and PCBs in the fish pellets**

	Control	C1		C2	
	measured concentration	targeted concentration	measured concentration	targeted concentration	measured concentration
PAH :					
fluoranthene	2.3	210	189	2100	2072
pyrene	3.2	200	176	2000	1916
benzo(a)anthracene	0.7	70	49.6	700	640
chrysene	1.5	130	84.8	1300	1144
benzo(b)fluoranthene	2.3	170	116	1700	1501
benzo(k)fluoranthene	0.7	60	43.3	600	529
benzo(a)pyrene	1.3	60	42.1	600	517
indeno(1,2,3-cd)pyrene	nm	50	nm	500	514
benzo(ghi)perylene	0	50	54	500	435
∑ PAH =	12	1000	754.8	10000	9268
CB :					
CB 28	0	5	4.2	50	47.1
CB 52	0.4	25	24.3	250	252
CB 101	1.6	50	43.8	500	470
CB 149	2.1	50	42.8	500	477
CB 118	1.1	50	41.7	500	465
CB 153	3.3	100	86	1000	939
CB 105	1.4	25	20.3	250	238
CB 138	2.6	100	79.4	1000	911
CB 156	1.2	25	20.3	250	218
CB 180	1.4	50	37.9	500	449
CB 170	1.5	25	17.7	250	212
CB 194	0	5	3.5	50	42.5
∑ PCB	16.6	510	421.9	5100	4720.6
total load of contaminants	28.6	1510	1176.7	15100	13988.6

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951 **Table 2. Primer sequences used for qPCR**

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Genes	Forward (5' to 3')	Reverse (5' to 3')	Origin
α-Tubulin	CAC-AGC-CTC-ACT-TCG-TTT-TG	AGA-TGA-CAG-GGG-CAT-AGG-TG	Leaver et al. (2010)
18S	GTC-TGG-TTA-ATT-CCG-ATA-ACG-AAC-GAG-ACT-CTA	TGC-TCA-ATC-TCG-TGT-GGC-TAA-ACG-CCA-CTT-G	Evrard et al. (2010)
CYP1A1	GCC-AAC-GTG-ATC-TGC-GGA-ATG	AAG-CCG-ACC-AGC-TCC-TGA-TC	Calves et al. (2011)
BHMT	AGA-GAG-GCC-TAC-AAG-GCT-GG	GTG-TGC-ATC-TCC-AGA-CCA-GCG-C	Evrard et al. (2010)
TNF-R	CAG-CCG-AAT-CTC-AGT-GAT-GG	CAG-TTG-GAT-GCC-AAG-TCA-GC	Designed from ES443667.1
C3	ACG-ATG-AAA-GTG-GGC-GTC-TT	TGC-AGT-TCT-CTT-CGG-CAC-AT	Designed from EC379465.1

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