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Responses of the European flounder *Platichthys flesus* to the chemical stress in estuaries: load of contaminants, gene expression, cellular impact and growth rate

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Abstract

European flounder responses to the chemical stress were assessed by a comparative approach on four estuaries displaying contrasted patterns of contamination. The contamination typology of the estuaries was investigated by individual measurements of contaminants in fish. Molecular and physiological responses were studied by gene expression, genotoxicity, neurotoxicity and growth rate. Fishes in contaminated estuaries were characterized by high levels of bioaccumulated contaminants, slow energetic metabolism and reduced growth rate, in contrast to the fish responses in the reference site. A seasonal effect was highlighted for contaminated flounder populations, with high PCB levels, high genotoxicity and elevated detoxification rate in summer compared with winter.

Keywords: *Flounder; estuaries; chemical contamination; bioaccumulation; biomarkers; gene expression*

Introduction

Coastal waters and particularly estuaries constitute key environments, as nurseries and reproduction zones for numerous fish or bird species. They are moreover demographically and economically active areas, and thus receive the various effluents of these activities (Kennish 1992, Abarnou et al. 2000, Matthiessen & Law 2002).

In this study, we focused on the fish responses to contrasted contamination patterns, along the French Atlantic coast, in the Vilaine, Loire and Gironde polluted estuaries and in a 'reference system', i.e. the weakly contaminated Ster estuary; these estuaries are localized in the Bay of Biscay.

It is difficult to characterize properly the chemical stress impacting the marine coastal environment, because of its temporal variability and the complexity of the contaminant mixture. In this study, the chemical analysis was aimed at characterizing contamination at the different sites, focusing on the major chemicals present in the fish tissues, i.e. several more or less persistent organic compounds and metals. Among organic contaminants, we chose to estimate the bioaccumulation of polychlorinated biphenyls (PCBs) in the livers of flounders and the biotransformation of polycyclic aromatic hydrocarbons (PAHs) by measuring the biliary content of a PAH metabolite. Furthermore, analysis of the liver metal content for three major elements, copper, lead and cadmium, was assessed in the different estuaries.

Fish display close physiological relationships to their environment as ectothermic organisms. Thus,

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they are sensitive to environmental disruptions, and particularly to chemical stress (Cossins & Crawford 2005). The European flounder (*Platichthys flesus*) is an estuarine species that resides during its juvenile stage in estuaries, being also tightly linked to estuaries at the subadult and adult stages; thus this fish species is considered to be a very good model organism for environmental monitoring (Hylland et al. 1996, Kirby et al. 2000). Moreover, the flounder is a benthic fish living in contact with the sediment, the major compartment for contaminant storage in the aquatic environment, and is thus particularly exposed to chemical stress (Koehler 2004, Minier et al. 2000).

In this article, the typology of contaminants was explored in fish tissues parallel to investigations on several biomarkers at different levels of biological organisation (molecular, cellular, organism levels), to test the efficiency of these tools in response to contrasted chemical contexts. Changes in gene expression are important components of acclimatization and/or adaptation to environmental changes (Schulte 2001, Gibson 2006, Marchand et al. 2006, Larsen et al. 2007). Transcriptome is highly dynamic and changes rapidly and dramatically in response to disruptions; therefore alteration in gene expression could be a good indicator of cellular responses to environmental stimuli and disturbances (Lockhart & Winzeler 2000). Transcriptomics, as a tool to underline early warning signals, can detect toxic effects before whole-organism effects become evident (Feder & Walser 2005). Several genes were selected, considering the following functions: energetic metabolism, detoxification and iron metabolism. Cytochrome c oxidase is an enzyme of the inner mitochondrial membrane. It forms complex IV of the mitochondrial chain, and catalyses the transfer of electrons from reduced cytochrome c to molecular oxygen (Barrientos et al. 2002, Carr & Winge 2003, Tsiftsoglou et al. 2006). ATPase is the ultimate complex of the respiratory mitochondrial chain that catalyses the production of ATP. CYP4501A1 is implied in the biotransformation of hydrophobic contaminants into water-soluble compounds, but also in numerous endogenous metabolic pathways (Flammarion 1997). Its induction by various xenobiotics explains its use as a pertinent biomarker in contaminated populations in the field (Williams et al. 2000). CYP4501A1 is particularly involved in the biotransformation of PAHs, the resulting molecules being sometimes more toxic than the parental ones (Van der Oost et al. 2003). Moreover, sex and reproductive status can modulate CYP1A1 transcriptional levels (Wirgin & Waldman 1998, Flammarion 1997). Betaine homocysteine methyltransferase (BHMT) catalyses the conversion of homocysteine to methionine (Ou et al. 2007) and thus plays a major role in the metabolism of amino acids. Its role as a detoxification enzyme in a pesticide context has been proposed by several authors (Marchand et al. 2006, Horst et al. 2007). Ferritin is a protein that stores iron, and thus protects the cell from damaging effects of free iron (Napier et al. 2005); it keeps iron sequestered in a bioavailable form and is thus implicated in the homeostasis of iron. This protein could play a major role in the cell protection against oxidative stress and hypoxia (Richardson 2003).

At the cellular level, several organic or inorganic chemicals can interact with DNA molecule, affecting its integrity. The level of DNA breaks induced by chemicals is thus considered as a precocious and sensitive biomarker for populations living in contaminated environments, particularly in aquatic systems (Winter et al. 2004, Alink et al. 2007). These primary damages could lead to carcinogenic effects or hereditary impacts, and could affect several biological traits linked to the population fitness, i.e. the reproductive success and the genetic structure (Newman & Clements 2008).

A second biomarker of cellular damage was investigated in this study: the neurotoxic effects of contaminants, focusing on the inhibition of the cholinesterases (ChE). Acetylcholine is the primary neurotransmitter in the sensory and neuromuscular systems in most species, and is essential to normal animal behaviour (Forget et al. 2003). The inhibition of acetylcholinesterase (AChE) is a sensitive biomarker for aquatic invertebrates and vertebrates exposed to organophosphorous and carbamate pesticides, and to a lesser extent to metals and PAH contamination (Payne et al. 1996, Guilhermino et al. 1998).

At the level of the organism, a fitness-related parameter was also investigated: the fish growth rate estimated by the analysis of otoliths. The growth rate is a sensitive indicator of the physiological status of an individual (Morales-Nin 2000). Growth is the result of the integration of many processes; thus its variation may represent the integration of sub-lethal responses to chemical contamination (Morales-Nin et al. 2007).

By the chemical analysis of selected PCB congeners, metals in liver and PAH metabolite in bile, and the use of a multilevel biomarker approach, the aim of this study was to assess pollutant exposure and effects in European flounders collected during two seasons (winter and summer) from four estuaries in the Bay of Biscay. Finally this study allowed us to explore the spatial and temporal variability of the fish responses to chemical stress in an estuarine context.

Materials and methods

Field collection

Study sites

Estuaries were selected in a coherent biogeographical unit, the Bay of Biscay (Figure 1). This choice enabled

Figure 1. Localisation of the sampling sites.

us to collect fish displaying a similar evolutionary history. Fish were sampled in three polluted estuaries (the Vilaine, Loire and Gironde) and in a 'reference' estuary (the Ster of Lesconil). The Vilaine catchment area spreads over one-third of Brittany and undergoes important impacts from agricultural activities. Thus, Vilaine waters are mainly contaminated by pesticides (Forget 1998, SAGE Vilaine 1999). Moreover, the waters are strongly impacted by eutrophication, leading to hypoxia crisis (Menesguen et al. 2001).

The Loire estuary displays a strongly urbanized and industrialized basin, and thus shows a diffuse contamination, characterized by a mixture of metals, PAHs, PCBs and pesticides (RNO 2003).

The Gironde estuary, the largest in Europe with an area of 635 km^2, shows a heavy chronic contamination, particularly with heavy metals such as cadmium (Durrieu et al. 2005). Furthermore, a recent research has underlined strong contamination by PCBs and polybrominated diphenyl ethers (PBDEs) in Gironde eels and flounders (Tapie 2006).

The Ster basin is considered as a reference site, because it receives low levels of domestic and agricultural effluents, and no industrial wastes (Marchand et al. 2003).

Fish sampling and sample preparation

Platichthys flesus were sampled during two periods of the year: the reproduction period (winter) and the sexual rest period (summer). A total of 65 fish were caught from the Gironde, the Loire and the Vilaine estuaries (35 in winter, 30 in summer for each estuary), and 58 from the Ster estuary (28 in winter and 30 in summer). Fish were sampled in January and July 2005 by trawling operations in contaminated estuaries (the Gironde, Loire and Vilaine), and in January and June 2006 by gillnets in the reference site (the Ster). Fish were collected at random in each estuary; their length ranged from 20 to 42 cm. The average sex ratio (males/females) and age (otolith observation) were estimated (pooled winter and summer data), respectively, for the Gironde, the Loire, the Vilaine and the Ster fish: 0.54 and 3.3 ± 0.2 years; 0.51 and 3.2 ± 0.2 years; 0.66 and 2.8 ± 0.3 years; 1 and 1.9 ± 0.1 years.

Fishes were anesthetized with MS-222 and blood was collected in heparinized syringes from the caudal vessels and transferred in heparinized test tubes. Blood samples were diluted (×100) in a cryopreservative buffer (250 mM sucrose, 40 mM trisodium citrate, 5% dimethyl sulfoxide (DMSO), pH 7.6, adjusted with 1 M citric acid), deep-frozen in liquid nitrogen and then stored at –80°C until processing. Liver and muscle were quickly dissected, bile was carefully collected and all tissues/organs were flash frozen in liquid nitrogen. Liver and bile for chemical analyses were carried to the laboratory and stored respectively at –20°C and –80°C. Liver and muscle samples intended for the investigation on biomarkers were carried to the laboratory and stored at –80°C until analysis. Otoliths were extracted from the skull, cleaned and preserved in plastic bag until use.

Chemical analysis

PCBs

Tissues were freeze-dried and crushed before analysis. Analysis was conducted on 30 fish by estuary. Each sample was extracted by microwave heating with dichloromethane as solvent (Thompson & Budzinski 2000) or by ASE as described by Tapie et al. (2008). The PCB measurement was made by spiking the matrix with internal standards before extraction as described by Tapie et al. (2008). PCB congeners 30, 103, 155, and 198 were used as internal standards. Samples were analysed by gas chromatography/electron capture detector (GC/ECD), using an HP 5890 series II gas chromatograph from Hewlett-Packard (Palo Alto, CA, USA) coupled to a ^{63}Ni electron-capture detector. PCB congeners analysed were CB 50 + 28, 52, 101, 118, 153, 138 and 180, representing the main PCBs found in the environment.

To test the accuracy and the validity of the quantification method, a standard solution of compounds to

be quantified mixed with the related internal standards were regularly run on the GC/ECD system. These solutions were used to calculate the response factors, while other independent solutions were used to test the precision of the quantification, evaluated to be more than 80% for all the tested compounds, with standard deviations between replicates less than 10% for all compounds. Moreover, the entire analytical procedure was applied several times to the certified mussel tissue SRM 2977 (NIST, Gaithersburg, MD, USA). The recoveries for three replicates on this SRM were between 80 and 120% depending on the compound, with a reproducibility <15%.

PAH metabolite: 1-hydroxypyrene

We measured the metabolite 1-hydroxypyrene (1-OHP) in fish gall bladder according to the method by Mazeas & Budzinski (2005). We evaluated the presence of PAH metabolites in the bile through the measurement of 1-OHP because of the wide presence of pyrene in the environment, its bioavailability, and its almost exclusive transformation to 1-OHP. Ten fish were analysed by estuary. Briefly, the method consisted in an enzymatic deconjugation (β-glucuronidase at 100 KU ml–1 and arylsulfatase at 7.5 KU ml–1) followed by a solid-phase extraction on a C18 cartridge and by a clean-up on an NH2 cartridge. The purified extracts were submitted to a derivatization step using bis(trimethylsilyl)-trifluoroacetamide (BSTFA; Acros Organics, Noisy-Le-Grand, France) before GC/mass spectrometry (MS) analysis, to enhance the separation and detection of compound. 1-OHP was detected as PAH-O-Si(CH3)3+, at the value m/z = molecular weight + 72.

Metal analyses

Metal analyses were conducted on 35 fish by estuary in winter (except for the Ster estuary, $n = 28$) and on 30 fish by estuary in summer. To measure metal concentrations, liver samples (70–100 mg wet weight) were dissolved in 2 ml nitric acid (65%, Suprapur; Merck, Whitehouse Station, NJ, USA). After dilution in 0.5 M NaCl (SigmaUltra; Sigma, St Louis, MO USA), concentrations of copper, lead and cadmium were assessed by stripping chronopotentiometric methods. These methods are detailed in Riso et al. (1997a, b) and were previously used for tissues (Chausson et al. 2001, Marchand et al. 2004, Tanguy et al. 2003). In order to check the accuracy of the method, various certified reference seawater samples were analysed (NASS-5, CASS-3 and SLEW-2, National Research Council of Canada). Analyses were made by spiking each sample three times with standards (Riso et al. 1997a, b).

Biological responses

Molecular responses: gene expression by RT-PCR

Molecular responses were analysed on between 28 and 35 fish, depending on the estuary and the season. Total RNA was extracted from the livers of *P. flesus* using a method based on extraction in guanidium isothiocyanate. In brief, livers were homogenized in guanidium isothiocyanate by using an Ultra Turrax T8 (IKA Labortechnik, Staufen, Germany) and sodium acetate (3 M), chloroforme (1/5 vol) and phenol (2 vol) were added. Samples were shaken and kept refrigerated for 15 min on ice. A centrifugation (12 000***g***, 20 min, 4°C) followed, and supernatant was recovered and precipitated with 2.2 vol of pure frozen ethanol. Samples were shaken and left at –20°C overnight. Total RNA was recovered by centrifugation (12 000***g***, 30 min, 4°C) and the pellet was washed with ethanol 70%. Finally, pellet was dried and resuspended in DEPC (diethyl pyrocarbonate) water. Optical densities (ODs) were measured at 260 nm using a spectrophotometer, and concentrations were quantified using 1 OD = 40 µg RNA. RNA integrity was checked by electrophoresis on agarose (1.5% in 0.5x TBE).

Reverse transcription was performed on 20 µg of total RNA, using oligodT primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). One microgram of cDNA was amplified in 2 mM of $MgCl_2$, and 4 pM of primers F and R for each gene (Table 1). Ribosomal 18S was used as the polymerase chain reaction (PCR) internal control, and amplified with 10 pM of primers F and R (Table 1). Sequences from GeneBank database were used to design some primers, as CYP4501Aa (AJ132353) and ferritin (DQ848938).

Table 1. Primers and conditions of amplification of genes used in the semiquantitative gene expression study.

Name	Forward primer	Reverse primer	T°C	Cycles
BHMT	5′-AGAGAGGCCTACAAGGCTGG-3′	5′-GTGTGCATCTCCAGACCAGCGCC-3′	55	45
Cytochrome c oxidase subunit 2	5′-AGCTACGAATACACAGACTACCA-3′	5′-ACGGCGTCTACTTTTACGCCCAG-3′	60	30
ATPase Fo subunit 6	5′-GAGATAAGGTAAAGCATTAGTGAGGC-3′	5′-CTACCCTGAACGCTCTTCCCAACCC-3′	62	30
Ferritin	5′-AAGAAACCGGCTCGTGATGA-3′	5′-GAGGGTCGTTGTGCTTGGA-3′	58	40
CYP4501A1	5′-GGCTTGGTGACCCTCAGTGA-3′	5′-CGGCTATTAATGCGCAAAAAG-3′	58	40
18S	5′-GTCTGGTTAATTCCGATAACGAACGAGACTCTA-3′	5′-TGCTCAATCTCGTGTGGCTAAACGCCACTTG-3′	58	30

BHMT, betaine homocysteine methyltransferase.

Primers of ATPase (CF379111), cytochrome c oxidase (CF379113) and BHMT (CF379280) were available at the laboratory of Marchand et al. (2006). The number of cycles and hybridization temperature were chosen after several developments to avoid signal saturation for each gene (Table 1). The resulting PCR products were electrophoresed in 0.5x TBE/1.5% agarose gel and visualized with ultraviolet radiation after staining with ethidium bromide. Images were analysed with the GeneProfiler software (Version 4.03, Scanalytics, Inc., Lincoln, NE, USA), each band corresponding to an OD. Results are given as the ratio of OD gene/OD internal control.

Cellular responses: genotoxicity assessment by the Comet assay

The comet assay was performed on flounder erythrocytes according to the procedure described by Singh et al. (1988) with slight modifications. Thirty fish were analysed by estuary and by season. Blood cells were rapidly thawed just before the comet assay; cell density was adjusted to 10^3 cells μl^{-1} of cold phosphate-buffered saline (PBS) and cell viability was checked by the Trypan blue exclusion method. In all experiments, the viability of cells was >95%. Microscope slides were first immersed in melted normal agarose prepared in PBS (0.8% final concentration) and dried overnight at room temperature. An aliquot of 50 µl of a 1% low melting point agarose in PBS mixed with an equal volume of cell suspension was spread on the slide and a coverslip added. After agarose solidification on an ice-chilled plate, a second layer was made with 90 µl of 0.5% low melting point agarose spread on the previous layer, a coverslip was added and slides were again allowed to solidify. All these steps were carried out under normal light but the following steps were carried out under red dim light to prevent artefactual DNA damage. The cells were treated with a lysing solution (2.5 M NaCl, 0.1 M ethylenediaminetetraacetic acid (EDTA), 10 mM Tris(hydroxymethyl)aminomethane (Tris), pH 10, 1% (v/v) 4-(1,1,3,3-tetramethylbutyl) phenyl-polyethylene glycol solution (Triton X-100) and 10% (v/v) DMSO) for 1 h at 4 C and then the DNA was allowed to unwind for 40 min in the electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH >13). The electrophoresis was run for 24 min at 20 V and 300 mA (0.6 V cm^{-1}). Slides were then neutralized using Tris buffer (0.4 M Tris, pH 7.5) and dried for 10 min in absolute ethanol. The DNA was stained with 0.05 mM ethidium bromide and scored using an Axioskop epifluorescence microscope (Zeiss, Germany) and the comet assay IV image analysis system (Perceptive Instruments Ltd., Haverhill, UK). Randomly selected cells from two replicate slides (50 cells per slide) were analysed. Among the different available parameters, the tail moment (product of tail migration and tail intensity) was measured for each cell. Comet figures that showed an extremely prominent tail lacking a clearly identifiable head were excluded from the analysis, as they can originate from necrosis or apoptotic processes (Fairbairn et al.1995).

Cellular responses: cholinesterase assay procedure (AChE activity)

Thirty fish by estuary and by season were analysed for cholinesterase activity. Muscle was homogenized (1/4 w/v) in phosphate buffer (0.02 M, pH 7) added with 0.1% triton X-100. The homogenate was centrifuged at 10 000***g*** for 20 min (4°C) and S9 supernatant was recovered. S9 supernatant was analysed with the method described by Bocquené & Galgani (1998) adapted to the microplate reader. Specific activity is expressed as nanomoles of AcSCh hydrolysed per minute per milligram of protein.

Response at the organism level: estimation of growth rate

Sagitta, the biggest otolith of the three available in fish, was used for the estimation of growth rate (GR) for each flounder (a total of 65 for the Gironde, Loire and Vilaine estuaries and of 58 for the Ster estuary). GR was estimated by back-calculation between the beginning of its first winter (L1) and the beginning of its second winter (L2) as described previously (Laroche et al. 2002, Marchand et al. 2003). In brief, three otolith parameters were measured through image processing (UTHSCA Image Toll v.2): the maximum radius or maximum distance from the nucleus to the periphery (R); the length at the beginning of the first winter (R1) and the length at the beginning of the second winter (R2). The fish length at different life times was estimated considering the relationship between the total length (Lt) of a fish and the total otolith radius R: $Lt = aR + b$ (with a and b constants). Thus, growth rate (GR) was back-calculated as: $GR = L2 - L1$.

Statistical analysis

Data were checked for normality using a Kolmogorov–Smirnov test. As gene expression, AChE activities and growth rate data were not normally distributed, nonparametric tests were applied. A Kruskal–Wallis ANOVA followed by a mean rank multiple comparison was carried out using Statistica 6.0 software package (Statsoft) to compare and classify means for each physiological marker. As the distribution of DNA damage measured by the comet assay did not follow a Gaussian distribution as described earlier (Bauer et al. 1998), both Kruskall–Wallis and Mann–Whitney nonparametric tests were used for data analysis.

In order (1) to compare estuaries according to season, contamination level and biological responses, and (2) to explore the possible relationships between parameters, a data matrix was prepared considering quantitative chemical and biological variables (13 quantitative variables × 16 mean individuals: four estuaries / two seasons / two sexes). This matrix was analysed by using principal component analysis (PCA).

Results

Chemical analysis

Organic pollutants

The sum of the eight PCBs was detected in the liver, and the 1-OHP concentration was detected in the gall bladder (Figure 2). In the case of PCBs, concentrations ranged from 190–900 ng g-1 liver dry weight. Estuaries can be classified according to their mean levels of detected PCBs (summer and winter pooled): the Gironde (872 ng g-1) > the Loire (586 ng g-1) > the Vilaine (358 ng g-1) > the Ster (260 ng g-1). The contamination profile is mainly dominated by PCB congener 153 (approximately 40% of total PCBs), PCB congener 138 (25%) and PCB congener 180 (18%). Within the Gironde, the Loire and the Ster, the highest concentrations of PCBs were found in fish collected during summer (Figure 2A). Conversely, fish collected in the Vilaine displayed higher PCBs concentrations in winter. High levels of 1-OHP (Figure 2B) were detected in fish collected in winter from both the Loire and Vilaine estuaries; an inverse trend being observed in the Gironde system, where a particularly high level of 1-OHP was clearly detected in summer. The levels of 1-OHP in the Ster estuary remained less than 50 ng g-1, whatever the considered season.

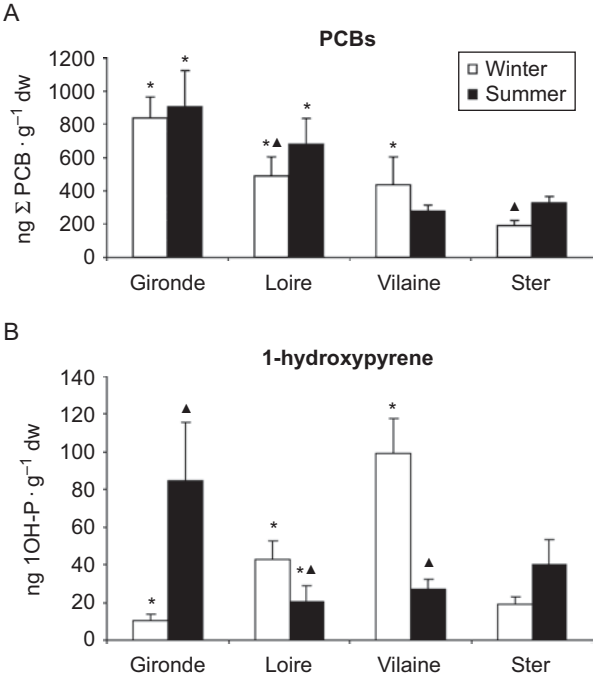

Figure 2. Mean (and 95% confidence interval) of organic contaminants in *Platichthys flesus* from the four Atlantic estuaries studied. (A) Polychlorobiphenyl (PCBs) concentrations (sum of 8 indicator PCBs) in *P. flesus* liver ($n=30$). (B) 1-hydroxypyrene (1-OHP) concentrations in *P. flesus* bile ($n=10$). *$p<0.05$ compared with Ster estuary, and (▲) for a significant difference between winter and summer.

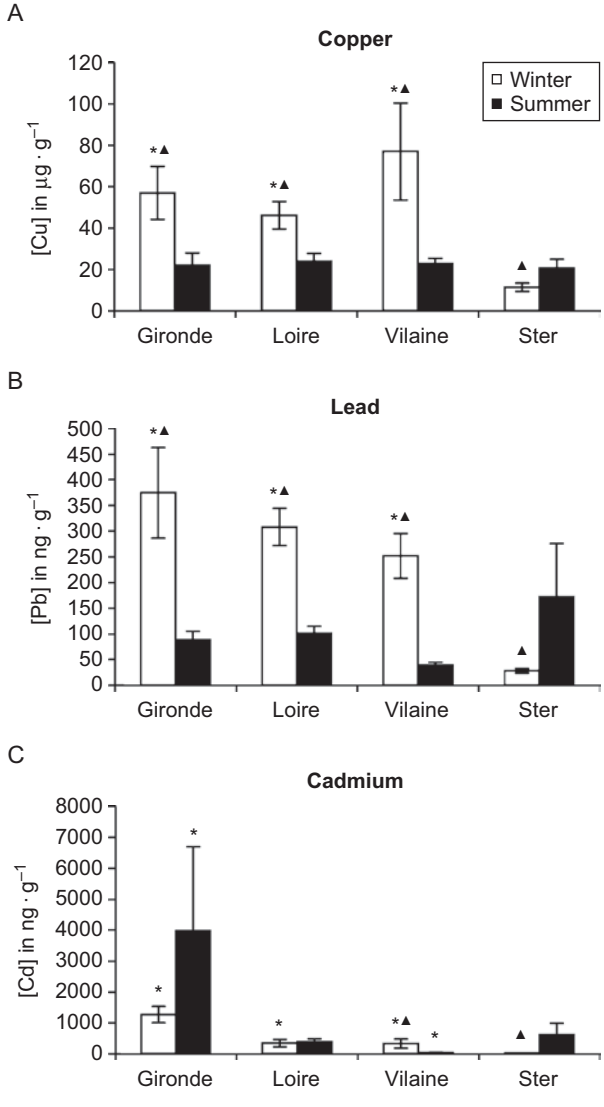

Figure 3. Mean (and 95% confidence interval) concentrations of heavy metals in *Platichthys flesus* liver from four estuaries of the Bay of Biscay. *$p<0.05$ compared with Ster estuary, and (▲) for a significant difference between winter and summer. (A) Copper concentrations in *P. flesus* liver ($n=35$ for winter, except for the Ster estuary where $n=28$, and $n=30$ for summer for each estuary). (B) Lead concentrations in *P. flesus* liver ($n=35$ for winter except for the Ster estuary where $n=28$, and $n=30$ for summer for each estuary). (C) Cadmium concentrations in *P. flesus* liver ($n=35$ for winter except for the Ster estuary where $n=28$, and $n=30$ for summer for each estuary).

Heavy metals

In general, Cu and Pb concentrations were lower in fish collected in summer compared with those caught during winter, except for the reference estuary (Ster) where an inverse trend was detected (Figure 3A, B). Copper concentrations ranged from 11 to 77 µg g^{-1} wet weight in liver (Figure 3A). In summer, limited variations in Cu levels were observed for all estuaries (i.e. 22 ± 6 µg g^{-1}). In winter, significant differences were detected between systems. In the Ster estuary, Cu levels were low (11 ± 2 µg g^{-1}) whereas in the Loire, the Gironde and the Vilaine, Cu levels were respectively of 46 ± 7 µg g^{-1}, 57 ± 13 µg g^{-1} and 77 ± 23 µg g^{-1}.

Pb levels in *P. flesus* liver (Figure 3B) varied between 28 and 380 ng g^{-1}. In summer, the lowest concentrations were encountered in the Vilaine estuary (39 ± 7 ng g^{-1}) and higher values were measured in the Gironde (89 ± 16 ng g^{-1}), the Loire (100 ± 14 ng g^{-1}) and the Ster system (170 ± 110 ng g^{-1}). In winter, the lowest Pb levels were observed in the Ster estuary (28 ± 4 ng g^{-1}), whereas in the other systems, the Pb levels were relatively high with values around 300 ng g^{-1}.

Cd concentrations in *P. flesus* liver from the various estuaries (Figure 3C) were measured within the range 17–4000 ng g^{-1}. Particularly high levels were observed in the fish from the Gironde estuary which is a system known to be affected by strong Cd inputs (Kraepiel et al. 1997). In the other estuaries, the lowest concentrations were found in the Ster estuary in winter (17 ± 5 ng g^{-1}) and in the Vilaine estuary in summer (38 ± 5 ng g^{-1}). As for Pb, it is worth noting relatively high Cd levels in the Ster estuary in summer (610 ± 380 ng g^{-1}).

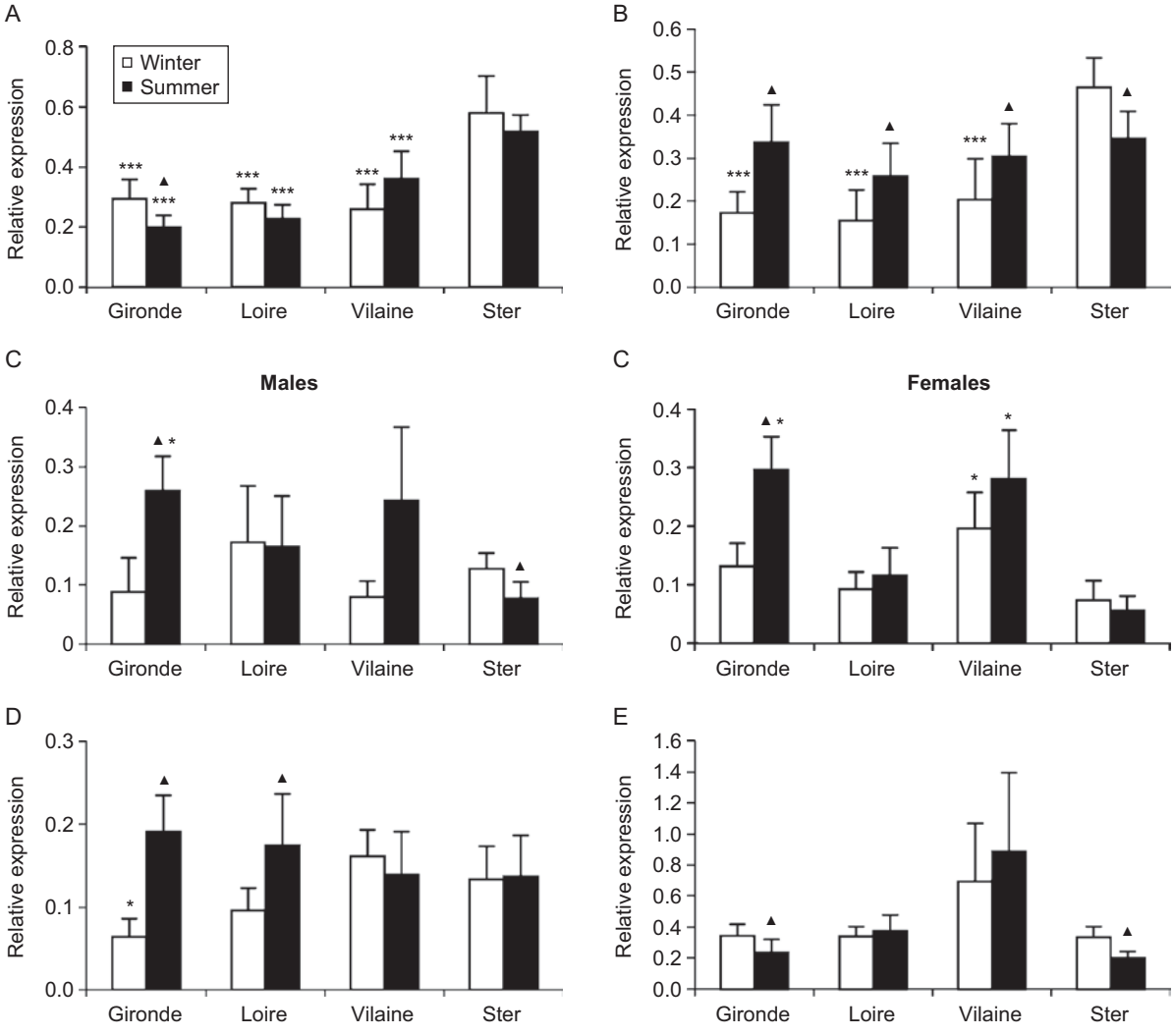

Figure 4. Relative expression of five transcripts in *Platichthys flesus* from different estuaries of French Atlantic coast. Expression is presented as the calculated ratio $OD_{transcript}/OD_{18S}$ after reverse transcriptase-polymerase chain reaction for $28 < n < 35$ individuals for each season. Means ± 95% confidence interval are presented. (A) Cytochrome c oxidase, subunit 2; (B) ATPase Fo subunit 6; (C) CYP450 1A1; (D) betaine homocysteine methyltransferase (BHMT); (E) ferritin. *, ***, significant difference with $p<0.05$ and $p<0.001$, respectively, compared with Ster estuary. (▲) for significant difference between summer and winter.

Gene expression

Gene expression was estimated by the relative expression of the transcripts according to sites (the Gironde, Loire, Vilaine, and Ster estuaries) and sampling seasons (winter/summer).

Cytochrome c oxidase subunit 2 (COX2) expression was significantly lower during winter and summer in the three contaminated systems (Figure 4A), compared with the Ster estuary ($p<0.001$). A difference in the relative expression of COX2 was detected between summer and winter within the Gironde estuary ($p<0.05$), but was not observed in the other systems.

ATPase expression was also significantly lower in winter in contaminated estuaries compared with the Ster estuary ($p<0.001$) (Figure 4B). In summer, no significant difference was observed between the Ster level and those of contaminated estuaries. ATPase expression was significantly different between summer and winter within each estuary ($p<0.05$). In contaminated estuaries, the level of transcription was higher in summer whereas an inverse trend was detected in the Ster estuary.

CYP4501A1 relative expression was presented according to the sex (Figure 4C). In winter, no significant difference in the CYP4501A1 transcription level was observed for males between all estuaries, whereas a difference was detected for females between the Ster and Vilaine estuaries ($p<0.05$). In summer, CYP4501A1 expression was higher for males in the Gironde estuary compared with the Ster while a higher transcription level was observed for females in the Gironde and the Vilaine estuaries compared with the Ster ($p<0.05$). A difference between winter and summer within the Gironde estuary was observed for male and female flounders, and also for males from the Ster estuary. Globally, CYP450 expression was higher in contaminated estuaries compared with the reference system.

A difference in BHMT expression was detected in winter between the Ster and the Gironde estuaries ($p<0.05$) but also between the Gironde/Loire group with respect to the Vilaine ($p<0.01$). On the other hand, no trend between contaminated and reference sites was detected in the summer (Figure 4D). A significant increase in BHMT expression ($p<0.05$) was observed from winter to summer, within the Gironde and the Loire estuaries.

No significant difference between contaminated and reference sites was observed for either season for ferritin relative expression (Figure 4E). Ferritin expression was higher in the Vilaine compared with other estuaries whatever the sampling season; however this increase was not statistically significant.

Genotoxicity assessment

Genotoxicity level in flounder blood was estimated by the single cell gel electrophoresis assay (comet assay) according to sites (the Gironde, Loire, Vilaine, and Ster estuaries) and to sampling periods (winter/summer). DNA damage level was expressed as tail moment values (Figure 5). During winter, discrepancies between sites remained low; however a significant but tiny difference in tail moment values was observed only in fishes from the Vilaine and Gironde estuaries that showed a 25% increase in DNA damage compared with the Ster. More contrast in differences between the sampling sites were observed in summer (Figure 5) when fishes from the Gironde, Loire and Vilaine estuaries exhibit a significantly higher genotoxic response than those from the Ster estuary ($p=0.024$, $p=0.011$ and $p=0.003$, respectively). For example, a 4-fold increase in tail moment values was measured in flounders from the Vilaine estuary compared with fishes caught in the Ster estuary (1.5- and 2-fold for the Gironde and Loire estuaries, respectively). Monitored DNA damage levels can be classified according to the following gradient: the Vilaine > the Loire > the Gironde > the Ster. A general trend toward an increase in DNA damage during summer was noticed, as tail moment values measured in June were 2–6 fold higher than in January, depending on the sampled estuary. No significant difference in DNA damage was detected between either sex, whatever the site or the sampling season (data not shown).

Cholinesterase response (AChE activity)

A seasonal comparison of the AChE activity showed a general reduction of activity for males and females

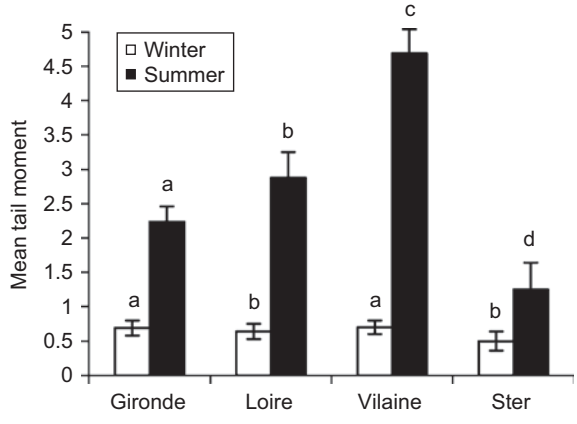

Figure 5. DNA damage expressed as mean tail moment in erythrocytes of flounder caught in different estuaries in winter and summer. Bars represent 95% mean confidence interval and letters show a significant difference between sampling stations at each season ($p<0.05$).

in winter compared with the summer, whatever the considered estuary (Figure 6). Significant differences were identified for females between winter and summer ($p<0.001$) only in the most contaminated sites: the Gironde and Loire. The temporal variation of the AchE activity was only significant for males in the moderately contaminated Vilaine estuary. Over the whole dataset, the lowest AChE activity was detected in winter for the Vilaine males ($p<0.05$) by comparison with the Ster estuary (Figure 6).

Assessment of growth rate

No significant differentiation was detected between males and females growth rate whatever the considered estuary; thus, the mean fish growth rate between the first and the second winter was estimated in the different estuaries, pooling males and females data (Figure 7). The classification of the average growth rates (GR) showed: GR Ster (163 mm/year) ≈ GR Vilaine (144 mm/year) > GR Loire (118 mm/year) ≈ GR Gironde (108 mm/year) with $p<0.05$.

Integration of biomarker responses

Integration of fish responses was analysed by PCA (Figures 8 and 9); the three main factorial axes explain, respectively, 28.7%, 23.6% and 19.5% of the total variance.

A group of variables detected on the right part of the correlation circle (axis 1 × 2: Figure 8A) and clearly linked to high bioaccumulation of particular pollutants (firstly Cd, PCBs and secondly Pb, Cu) was opposed to variables on the left part of the circle displaying a strong expression of cytochrome c and a high growth rate. Thus, in the factorial plan 1 × 2 (Figure 8B), a clear opposition was detected between: (1) contaminated systems on the right (particularly the Vilaine and Gironde in winter) where fishes were characterized by a significant load of bioaccumulated contaminants, reduced growth rate and slow energetic metabolism, and (2) reference system on the left (the Ster), where fishes displayed weak contamination and higher growth rate and energy production.

In the upper right part of the correlation circle (axis 1 × 2: Figure 8A) a group of variables was characterized by high expression of BHMT and CYTP450, strong AChE and high level of 1-OHP; this group was linked to the Gironde estuary in summer (Figure 8B). In the opposite position of the correlation circle, i.e. in its lower left part, fishes displaying high ferritin expression were clearly detected in the Vilaine estuary in summer (Figure 8B).

On the upper part of the correlation circle (axis 2 × 3: Figure 9A) a group of variables was highly correlated with axis 3 and was characterized by pronounced DNA damage, high expression of CYTP450 and high level of PCBs; this group was apparently linked to the contaminated Gironde, Loire and Vilaine estuaries in summer (Figure 9B). Moreover, the lower part of the factorial plan 2 × 3 (Figure 9B) was mainly characterized by contaminated estuaries sampled in winter; this seasonal pattern was not detected for the reference Ster estuary.

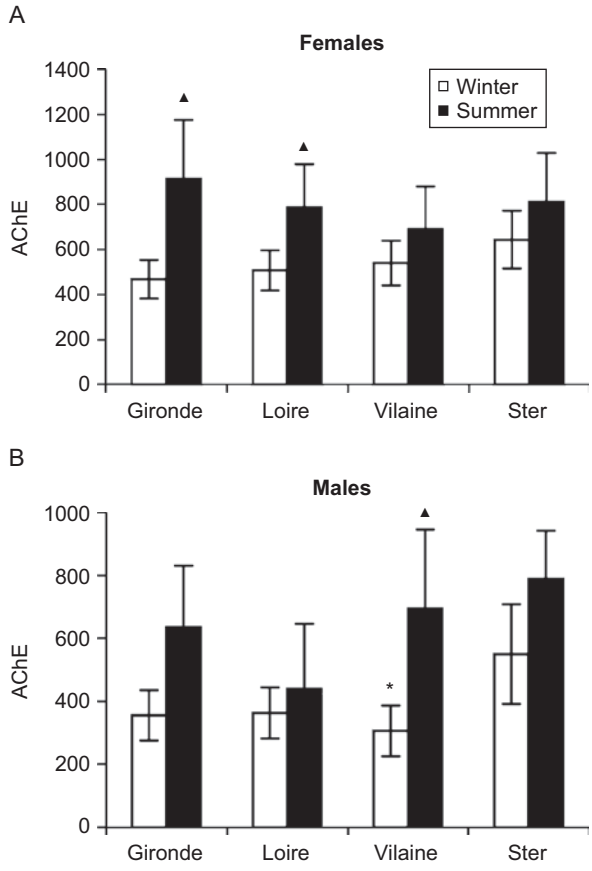

Figure 6. Mean acetylcholinesterase enzymatic activities in *Platichthys flesus* muscle from four estuaries of the French Atlantic coast. Confidence intervals are presented. *, significant difference with $p<0.05$ compared with Ster estuary. (▲) for significant difference between summer and winter.

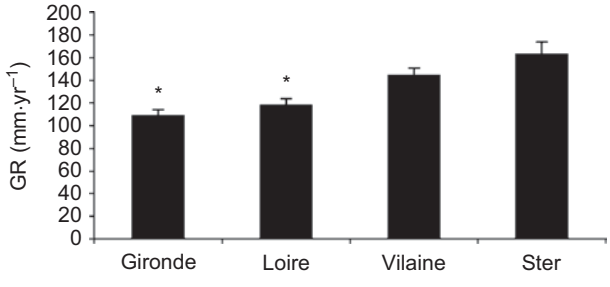

Figure 7. Mean growth rate (GR) (mean ± 95% confidence interval). *, $p<0.05$ compared with Ster estuary.

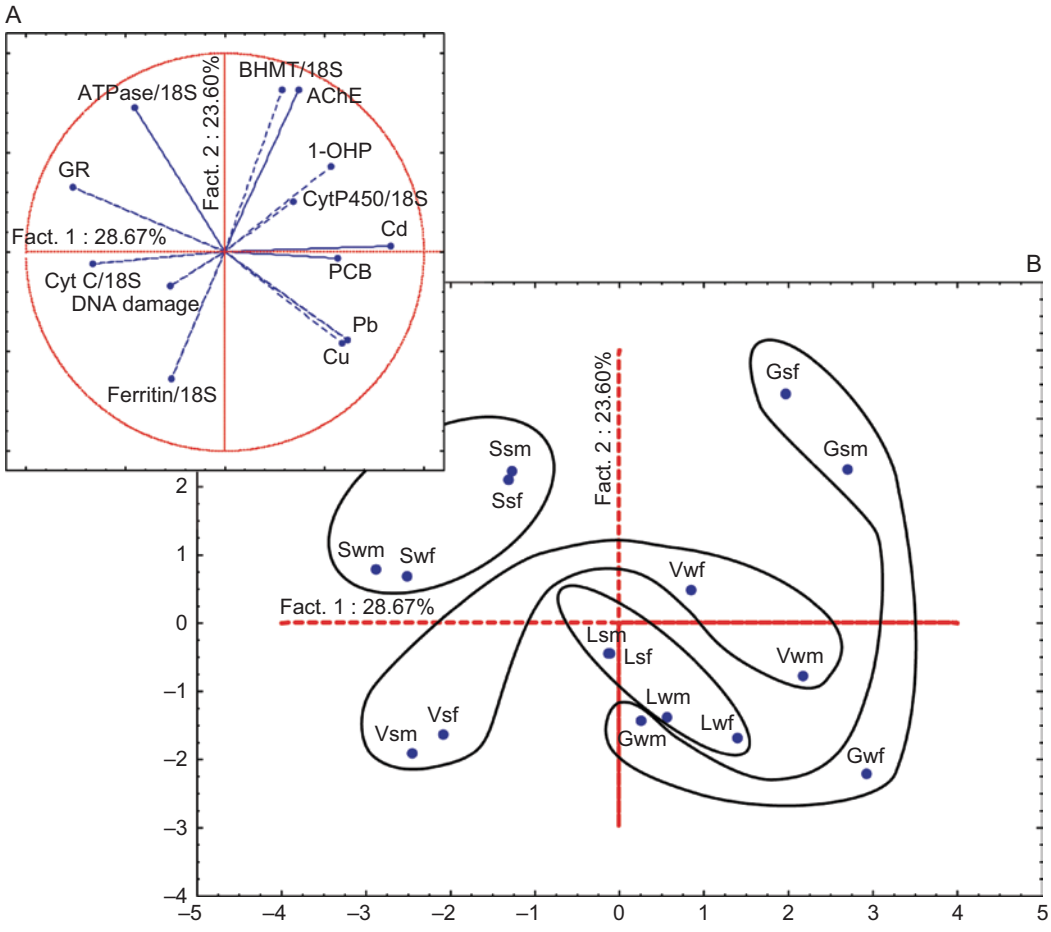

Figure 8. Principal component analysis on factorial axes 1 and 2. (A) Projection of quantitative variables on the correlation circle. (B) Projection of individuals on the factorial axis.

Discussion

The difference in the PCB levels in fish between estuaries has already been explored in a previous work (Tapie 2006) where a decreasing gradient of PCB contamination was detected in flounder liver over the different estuaries in the Bay of Biscay: the Gironde > the Loire > the Vilaine > the Ster. The current results confirm those previously reported by Tapie (2006) and also underline the 'low contamination' status of the Ster estuary with regard to PCBs.

In this study, the Ster estuary appeared to be the system least contaminated by PAHs. It can be considered as a reference site according to the baseline level defined by Budzinski et al. (2004), during the Erika survey of sole contamination (50 ng g^{-1} for 1-OHP). According to this baseline, two sites appeared as significantly contaminated by PAHs: Gironde in summer and Vilaine in winter; the contamination could be chronic in the Vilaine estuary but could be accidental in the Gironde estuary.

The seasonal differences in *P. flesus* liver metal content could be mainly explained by (1) the freshwater input variability observed in estuaries along the seasonal cycle, and (2) the metal speciation in estuarine waters linked to the salinity (Waeles et al. 2005, 2007). Thus, the metal content of a given area is potentially higher in winter compared with the summer and could explain the elevated Cu and Pb concentrations in *P. flesus,* for the polluted systems in winter. On the other hand, the dissolved Cd behaviour in estuarine areas is characterized by strong positive deviations from theoretical dilution lines. Thus, the highest cadmium levels in estuaries can be found over a wide salinity range (10–25) and the prediction of seasonal variations of the Cd content for a given area, as for Cu and Pb, is no longer possible. Moreover, important Cd inputs from sediment can happen in estuaries; these inputs were highly variable along the system itself and through the seasonal cycle (Monbet 2004), thus the interpretation of Cd variations in *P. flesus* liver could be particularly difficult.

Globally, chemical results in flounder liver allowed us to consider the Ster estuary as a reference site displaying a low level of contaminants compared with the Gironde, Loire or Vilaine estuaries. Furthermore, an inverse temporal trend was observed for the contamination in the

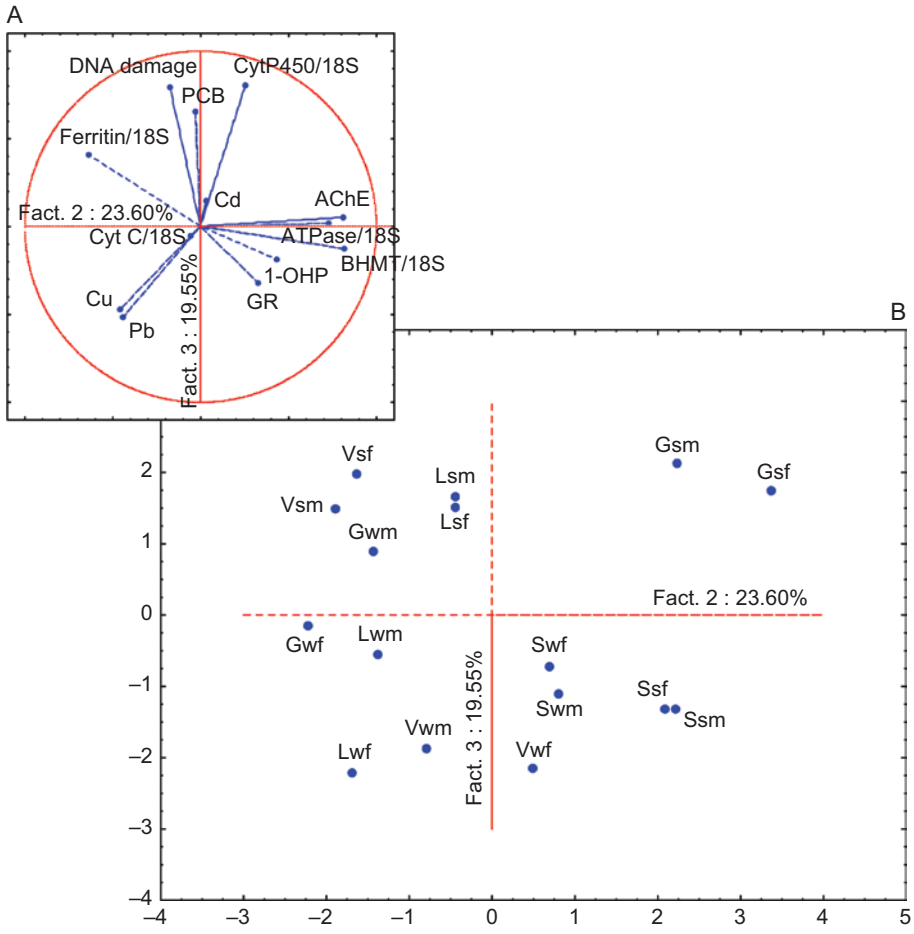

Figure 9. Principal component analysis on factorial axes 2 and 3. (A) Projection of quantitative variables on the correlation circle. (B) Projection of individuals on the factorial axis.

reduced Ster estuary compared with the large polluted systems, the concentration of metals being higher in summer compared with winter. This original pattern of the Ster system was probably linked to the original hydrological conditions characterizing the reduced pool where fish are collected during ebb tide, particularly in summer.

In the present study, we used semiquantitative RT-PCR to assess flounder gene expression in three contaminated estuaries and a less contaminated one. Among the transcripts chosen, two were involved in energy metabolism: COX2 and ATPase. We observed a significant downregulation of COX2 expression in winter and summer in the three contaminated estuaries compared with the Ster estuary. These results suggest a decrease of COX2 transcription linked to the chemical stress. Cytochrome c oxidase complex IV is an electron-transferring protein that contains heme as a prosthetic group. COX plays a key role in respiration, being responsible of more than 90% of oxygen consumption by organisms (Xu et al. 2005). Complex IV is made of subunits that are encoded by both nuclear and mitochondrial genomes. The assembly of these subunits and thus the activity of COX could be interrupted by a heme deficiency (Atamna et al. 2001). This could promote the collapse of mitochondrial membrane potential, oxidative stress, and the release of cytochrome c from mitochondria (Tsiftsoglou et al. 2006). All these events could finally induce cell death (Ye & Zhang 2004), and affect cellular respiration, thus leading to a lack of energy sources (Tsiftsoglou et al. 2006). The downregulation of this transcript in fishes stemming from contaminated estuaries could show a lower capacity of these populations to maintain a 'normal' production of energy at the cellular level, compared with a population submitted to a limited chemical stress.

In winter, the downregulation of COX2 observed in polluted populations is strengthened by the downregulation of ATPase, revealing a wrong functioning of the mitochondrial electron transport chain. ATPase, the last complex of the respiratory chain, catalyses the formation of ATP, thus its downregulation implies that less ATP is produced via the mitochondria.

No significant difference between winter and summer in the gene expression for the previous two genes

(COX2, ATPase) was also observed for adult flounders in a previous work in the reference system (Ster); whereas some temporal variation for the expression of these genes was detected in the Vilaine estuary (Marchand et al. 2006). Temporal variation of gene expression results from the complex interactions between: (1) the effect of temperature on the fish general metabolism, (2) the physiological status of the organism, and (3) the impact of environmental multistress. For the flounder in a reference estuary (i.e. without major stress), the reduced metabolism linked to the low environmental temperature in winter is probably compensated by the necessity to maintain a high metabolic rate to produce the fraction of energy allocated to reproduction which is considerable for *Platichthys flesus* in winter (Van der Veer et al. 2001). The reproduction period could make the responses of biomarkers more complex (Goksøyr et al. 1996).

Finally, the monitoring of the two transcripts (COX2 and ATPase) in polluted estuaries could produce a pertinent signal to follow a possible disruption of energy metabolism. The possible reduced level of expression of these genes means, for contaminated fish, probably less energy is available for higher needs linked to the cellular protection/detoxification effort. This situation could lead to a more rapid fish exhaustion affecting health status. In the future, a biochemical approach should be conducted on these genes to verify if the reduced expression is correlated with a reduction of the enzymatic activity.

In this study two transcripts were involved in detoxification processes: CYP1A1 and BHMT. The CYPs can metabolize endogenous and exogenous compounds, and their substrates in the liver include drugs or toxic compounds, as well as breakdown products. CYP1A1 is involved in phase I xenobiotics and drug metabolism (Williams et al. 2000). BHMT has been proposed by some authors to be involved in phase II of detoxification, in particular of herbicides (Marchand et al. 2006, Horst et al. 2007). Globally, the relative upregulation of CYP4501A1 observed here for both seasons in the contaminated estuaries compared with the 'reference' estuary has been traditionally observed in the literature (Wirgin & Waldman 1998, Williams et al. 2003, George et al. 2004). Nevertheless, trends are slightly different among estuaries.

Fish from the Gironde showed a pattern of upregulation for the CYP1A1 transcript in summer. The Gironde is relatively impacted by pollution, as shown previously by the level of PCBs, PAH metabolite and cadmium detected in summer fish tissues. This diffuse and complex contamination could explain the increased rate of transcription of this detoxification gene. In particular, it could mirror the increase rate of metabolism of organic contaminants, as observed by the especially high concentrations of 1-OHP in summer. In the Vilaine estuary, CYP1A1 was upregulated particularly in summer. This upregulation here may not be related to PAH transformation, because 1-OHP concentrations were low in summer. As the Vilaine estuary is predominantly contaminated by pesticides in summer (Marchand et al. 2006), the observed CYP1A1 upregulation along this season could be more related to pesticides than to other chemicals. The Vilaine situation in winter was specific; only females showed a higher CYP1A1 transcription that could be related to the high levels of 1-OHP detected. Surprisingly, males did not show a CYP1A1 differential expression compared with the Ster estuary. The hypothesis could be an interference with reproduction, which could hide the detoxification signal. Nevertheless, this interference is generally observed for mature females (Goksøyr & Förlin 1992, Flammarion et al. 1998, Flammarion & Garric 1999). The reasons for such an opposite trend remain unknown.

BHMT relative expression in winter in the Gironde and Loire estuaries was globally downregulated compared with the Ster, in contrast to its relative upregulation in summer in both systems. Nevertheless, the seasonal pattern observed for this transcript in the Gironde and Loire estuaries could be linked to the variation of confounding factors (temperature, salinity, dissolved oxygen) more or less related to the modification of the bioavailability of contaminants in the system.

Ferritin was upregulated particularly in the Vilaine estuary for both seasons, compared with the other estuaries. Ferritin is a protein that stores iron in the cytoplasm to protect cells from the damaging effects of free iron and can contribute to the defence of cells against hypoxia effects. This complex ferritin–iron is also a means of keeping iron in a bioavailable form for the cell (Napier et al. 2005). Iron plays a crucial role in mitochondrial metabolism, in particular for heme biosynthesis.

The Vilaine estuary exhibited periodically hypoxic conditions during summertime, mainly induced by eutrophication and by the retention/leak of waters by a dam localized in the upper part of the estuary (Menesguen et al. 2001). Moreover the particularly dry and hot summer 2005, might have favoured the closure of the dam, and thus a reduced flow of contaminants in the estuary. Thus, we suggest that in summer 2005, the chemical stress was probably low compared with the hypoxic stress in the Vilaine estuary; nevertheless, we can not reject the influence of other chemicals (pesticides, pharmaceuticals, etc.) on fishes, considering the difficulty in properly characterizing such compounds in fish tissues. Moreover we suggest that a possible hypoxic crisis could also appear in winter at the bottom level, particularly during neap tides. Finally we suggest that the ferritin overexpression detected in the Vilaine estuary in this study, could be more related to oxygen depletion than to chemical stress.

This study highlights the use the comet assay for DNA damage assessment in flounder erythrocytes, as previously reviewed during the past decade in other aquatic organisms from freshwater or marine environments (Mitchelmore & Chipman 1998, Lee & Steinert 2003, Frenzilli et al. 2009). Several advantages of this technique can be presented: the possibility of measuring DNA damage at the single cell level in most eukaryotic cell types, the large array of primary DNA damage that can be assessed (single- and double-strand breaks stemming either from direct or indirect acting compounds, alkali-labile sites, abasic sites, cross-links, unstable DNA adducts), and both the high sensitivity of the technique and the possibility of measuring early cell response. Moreover the comet assay is an easy-to-use approach when carried out on cells from fish biological fluids such as blood or sperm (Devaux et al. 1998, Labbe et al. 2001, Larno et al. 2001, Zhou et al. 2006).

Whatever the sampling season, fish from the Ster estuary exhibited less DNA damage than those from the other estuaries, and the highest genotoxic response was measured in fish from the Vilaine estuary. As stated above, the Ster estuary remains weakly contaminated compared with the other estuaries. In contrast, the Vilaine estuary is known to be under a pesticide pressure (Marchand et al. 2006), which could account for the highest genotoxic response observed in flounders living in this estuary. Although some pesticides have been clearly described as potent genotoxicants, studies aimed at measuring genotoxic damage in fish exposed to pesticides through the comet assay have often been restricted either to the assessment in laboratory controlled conditions after acute exposure (Ateeq et al. 2005, Pandey et al. 2006, Sharma et al. 2007) and/or to freshwater ecosystems (Bony et al. 2008). Unfortunately, no pesticide analyses in fish are available in this study due to the difficulty in properly analysing complex mixtures of pesticides in fish tissues.

Moreover, the significant increase in DNA damage measured in erythrocytes of flounders caught in the Vilaine, Loire and Gironde estuaries compared with the Ster cannot be easily explained by the results of the chemical analyses carried out in flounder liver. Only fish from the Gironde estuary showed a significantly higher contamination regarding the concentration of Cd in fish liver (both seasons) and PCBs in liver (both seasons), compared with other stations. This could partially account for the observed genotoxic response, although if Cd is a well-known genotoxicant in fish through reactive oxygen species (ROS) formation, PCB genotoxicity remains more controversial, depending on the family compound (Belpaeme et al. 1996, Risso-de Faverney et al. 2001, Barsiene et al. 2006).

Lack of difference in DNA damage levels measured in male and female flounders both out of and during the reproductive season suggests that sex does not interfere with the genotoxic response. As far as we know such a link has not been demonstrated to date and the present results are in agreement with previous studies carried out in other fish species (Devaux et al. 1998, Flammarion et al. 2002).

An hypothesis can be put forward to explain the observed difference in DNA damage between winter and summer. The high temperatures of estuary water monitored during summertime can lead to physiological stress in flatfish through a significant decrease of dissolved oxygen content of the water; this reduction of oxygen availability could be amplified in a eutrophicated system like the Vilaine estuary, by the bacterial degradation of organic matter at the water/sediment interface. To counteract such oxygen depletion, fish adapt themselves by overbreathing, thus modifying oxidative status. Moreover, fish overbreathing can increase pollutant intake by gill route. Both can give rise to higher DNA strand break production as previously described in fish (Liepelt et al. 1995). Finally DNA damage can be enhanced by thermic shock itself as described by Barsiene et al. (2006) in fish and shellfish.

Over the whole dataset, the lowest AChE activity was determined for the male fishes in winter, in the Vilaine estuary. Kopecka and Pempkowiak (2008) also demonstrated a greater inhibition of AChE activity in male flounder in the Baltic Sea. Reduced AChE activities were also observed for the female fish in winter, but the lowest levels were detected for the Gironde and Loire systems. In summer, the lowest activity for females was observed in the Vilaine estuary. The seasonal variation of the AChE was particularly contrasted in the Vilaine estuary and could be linked to combined effects of hypoxia and chemical stress.

The AChE activity measured in estuaries confirmed that the temperature is a critical environmental parameter for this activity (Bocquené & Galgani 1998); a general increase of the AChE activities was detected for the flatfishes, from winter to summer (Kirby et al. 2000, Hylland et al. 1996). Furthermore, within the same estuary, sex differences were also evident when considering this AChE activity.

If the comparison of AChE activities between different populations must be discussed cautiously, considering possible season and sex effects, the particular status of the Vilaine estuary in this study could indicate that possible inhibition of this activity could be linked to the specific cocktail of chemicals in this estuary. Nevertheless, this trend should be confirmed for the Vilaine system in the future, probably on a fish tissue displaying a higher sensibility to neurotoxicity, i.e. the brain.

A decrease of the flounder growth rate was detected in the most polluted estuaries (Gironde, Loire) compared with the moderately contaminated systems (Vilaine,

Ster). This trend was also highlighted in previous works on the flounder (Marchand et al. 2003, 2004). This reduced growth rate could be the result of the energy cost of detoxification/protection/restoration processes, commonly involved in coping with the chemical stress. This allocation of energy to toxicant response reduces the available energy for several life history traits, such trade-offs being described in the literature (Gimeno et al. 1995, Van Straalen & Hoffman 2000, Durou et al. 2007, Pierron et al. 2007). Nevertheless, this hypothesis must be considered cautiously because variables other than chemical contamination can interfere with growth (temperature, salinity, food availability, etc.).

Principal component analysis allowed classification of the main factors explaining the spatial and temporal variability of the chemical and biological signals, detected in flounder populations stemming from contrasting environmental contexts. The main factor was clearly linked to the contamination gradient which separated: (1) the reference population displaying high growth rate and elevated metabolic rate (i.e. Ster) from (2) the contaminated populations showing bioaccumulated pollutants (metals and PCBs), reduced growth rate and limited metabolic rate (i.e. Loire, Vilaine, Gironde in winter).

The second factor underlined the particular status of two polluted populations: (1) the Gironde in summer where fishes displayed high activities of detoxification and particularly of biotransformation of PAHs, resulting in elevated level of 1-OHP and (2) the Vilaine in summer where fishes were characterized by a particularly high transcription level of ferritin, probably linked to hypoxic stress.

The third factor was related to a seasonal effect opposing: (1) the populations of Vilaine, Loire and Gironde sampled in summer and displaying high PCB bioaccumulation and elevated levels of detoxification and genotoxicity, to (2) the populations of the same estuaries sampled in winter, showing decreasing values for the previous parameters.

Furthermore, the presumed weakly contaminated site of this study, the Ster estuary is mainly characterized by low levels of metallic and organic contaminants in the flounder liver, and by a reduced temporal variability of the biological responses; thus it could be considered as a valid 'reference site' for future ecotoxicological studies in the Bay of Biscay.

More generally, this multi-estuary approach highlights the pertinence of the European flounder *P. flesus* as a sensitive model for the monitoring of estuarine systems, as this species displays differential chemical and biological signals in contrasting estuaries. Within contaminated systems, the temporal variability of the chemical signals detected in the fish tissue was high and related to variable bioavailability of pollutants in the field, hydroclimatic parameters, level of metabolic activity, fish physiological status, etc.; this temporal variability should be considered more thoroughly in the future, to describe more accurately the typology of the chemical stress in coastal zones.

The use of several markers reflecting different levels of biological organization (from the molecular to the individual levels) allowed (1) identification of particular mechanisms linked to chemical stress (detoxification), (2) exploration of possible responses to multistress (survey of energetic metabolism, response to hypoxia and/or to thermal stress), and (3) also measurement of more ecologically relevant parameters such as growth rate, in more or less-disturbed environments, the chemical stress being one of the components of the multistress impacting fish populations in estuaries.

This work focused on fish populations chronically submitted to a more or less-pronounced multistress conditions in their natural environment; within each estuary, new investigations are currently in progress to explore the possibility of local adaptation developed by these populations to cope with stress. Thus, the interindividual responses to chemical stress was analysed, adding the genetic variability for several candidate genes; previous explorations on genotype–phenotype coupling on flounder populations showed possible resistance of particular genotypes to the chemical stress (Laroche et al. 2002, Marchand et al. 2003, 2004).

The analysis of the responses of natural flounder populations in their environment should be completed in the future by new strategies to characterize the pollution impact on estuary biota; one pertinent approach could be to expose fish collected from a control area to sediments from polluted estuaries under laboratory conditions and to examine the time-course variations of biomarkers, without the confounding factors of the estuarine conditions.

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