Science of The Total Environment
December 2019, Volume 695, Pages 133700 (13p.)
https://doi.org/10.1016/j.scitotenv.2019.133760
https://archimer.ifremer.fr/doc/00509/62067/

Coupling caging and proteomics on the European flounder (*Platichthys flesus*) to assess the estuarine water quality at micro scale

Borcier Elodie [1], Artigaud Sebastien [1], Gaillard Jean-Charles [2], Armengaud Jean [2], Charrier Gregory [1], Couteau Jérôme [3], Receveur Justine [4], Ouddane Baghdad [5], Diop Mamadou [6], Amara Rachid [6], Laroche Jean [1], Pichereau Vianney [1, *]

[1] Univ Brest, CNRS, IRD, Ifremer, LEMAR, F-29280 Plouzane, France
[2] Laboratoire Innovations Technologiques pour la Détection et le Diagnostic (Li2D), Service de Pharmacologie et Immunoanalyse (SPI), CEA, INRA, F-30207 Bagnols-sur-Cèze, France
[3] TOXEM - Le Havre, France
[4] CEDRE – Brest, France
[5] LASIR UMR 8516 – Lille, France
[6] LOG UMR 8187 – Wimereux, France

* Corresponding author : Vianney Pichereau, email address : vianney.pichereau@univ-brest.fr

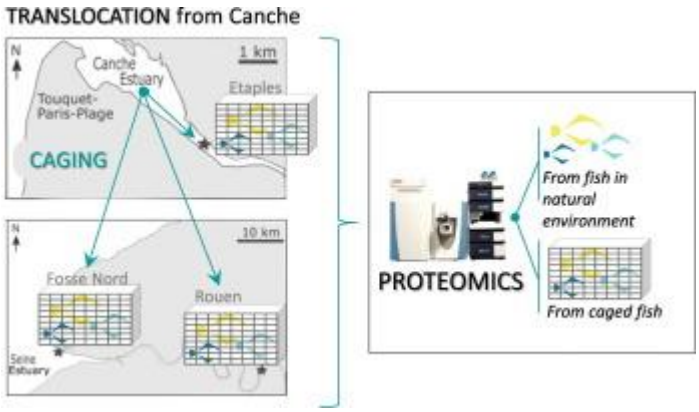
Abstract :

Estuaries are important areas highly vulnerable to anthropogenic pollutions. Therefore, the assessment of estuarine water quality is a major ecological issue. In this study, we sampled juveniles of the European flounder in the “pristine” Canche estuary, and caged them in Canche and in two polluted sites of the Seine estuary, Rouen and Fosse Nord. After one month, the metal and organic pollutants in these sites were assessed, and we evaluated several phenotypic indicators (condition index, RNA/DNA ratios and genotoxicity), and extracted the proteins in fish livers for analysis using a shotgun proteomics approach.

The results showed strong modifications in the fish caged in both sites of the Seine estuary, as compared to those caged in Canche. In particular, many proteins involved in phase I and phase II detoxification reactions were accumulated in the liver of fish caged in the site showing the highest pollution, Rouen. In addition, we observed a general disruption of metabolism, in particular an increase in lipid synthesis and carbohydrate degradation in Rouen, and a decrease in the abundance of proteins associated to translational activity in Fosse Nord. At both sites, several stress proteins were decreased.

The proteomic impact of the encagement by itself was also evaluated, by comparing the liver proteome of fish caged in Canche to that of fish stayed in natura during the same time. The results showed proteomic signatures of exposure to stressful conditions (particularly heat stress), most probably related to the micro-habitat in which the cages were placed. In conclusion, the caging technique is of great interest for ecotoxicological assessment of estuarine waters, but should consider that the results are representative of the micro-habitat around the cages, which does not necessarily represent the overall heterogeneity of the estuarine environment.

Graphical abstract



Highlights

► *Platichthys flesus* juveniles were caged in two differentially polluted sites of the Seine estuary. ► The liver proteome of fish phenotypes were determined. ► Proteins implied in xenobiotics detoxification were positively deregulated in the most polluted area. ► Control fish (caged in Canche) overexpressed many stress proteins. ► Caging is an interesting technique to evaluate estuarine water quality at a micro-scale.

Keywords : Estuary water quality, Ecotoxicology, Caging, Stress, Fish physiology, Shotgun proteomics

Introduction

Estuaries are among the most productive ecosystems over the world, giving them a high ecosystemic value (Costanza *et al*., 1997; Courrat *et al*., 2009). These coastal ecosystems are generally considered as major nurseries for many marine species (Chapman & Wang, 2001). Nowadays, estuaries are heavily impacted by anthropogenic activities, such as resource overexploitation, pollution, or habitat fragmentation (Aubry & Elliott, 2006; Lotze *et al.,* 2006). Monitoring the overall quality of estuarine habitats, especially those acting as fish nurseries, is therefore of utmost importance to preserve marine resources (Beck *et al.,* 2001).

The Seine estuary is one of the most anthropogenically polluted estuaries in Europe (Carpentier *et al.,* 2002; Cachot *et al.,* 2006; Burgeot *et al.,* 2017). Its basin covers more than a quarter of the French population and includes major harbours, industrial and agricultural areas (Mouny & Dauvin, 2002; Dévier et al., 2013; Erraud et al., 2018). Thus, the Seine estuary is subjected to eutrophication, organic and metallic contaminations (Blanchet et al., 2008; Dauvin, 2018). Furthermore, this estuary is particularly concerned by degradation and loss of flatfish nursery habitats, thus displaying a reduced capacity for the recruitment and growth of juvenile fish (Riou *et al.,* 2001, Le Pape et al. 2007; Rochette *et al.,* 2010; Archambault *et al.,* 2015; Tecchio *et al.,* 2016).

The quality of nursery can be measured by density, survival and growth of juveniles, and by the possibility for fish to move towards subadult habitats (Beck *et al.,* 2001; Schloesser & Fabrizio, 2018). The growth and survival of fish depend directly of prey availability and predation, but also of hydrological conditions and anthropogenic factors (Amara et al., 2007, 2009). A large panel of pollutants (metals, PAHs, PCBs, …) are monitored in the Seine estuary, and numerous studies explored the resident wild life responses to xenobiotics in this highly polluted ecosystem (Ohe *et al.,* 2004; Marchand *et al.,* 2004; Galland *et al.,* 2013; Dupuy

et al., 2015; Borcier et al., 2016; Erraud et al., 2018). In order to monitor the health status of fish *in natura*, many studies have measured biomarkers as cost-effective, sensitive and early warning signals of pollution in aquatic ecosystems (Amiard-Triquet and Berthet, 2015). Thus, classical biomarkers were carried out on Seine fish to detect DNA damages (Comet test, DNA adduct), neurotoxicity (AChE assay), detoxification process (EROD), and endocrine disruptions (Intersex, VTG levels) (Minier et al., 2000; Minier and Amara, 2008; Burgeot *et al.*, 2017).

High throughout shotgun proteomics offers the unique possibility to examine, in a single experiment, the thousands of proteins enabling the cells, organs and organisms, to adapt to abnormal situations. In the particular case of ecotoxicoproteomics, we can gather information on both the overall physiology of organisms subjected to xenobiotics, but also obtain a global, quantifiable biochemical, physiological, and/or "organismal-level" view reflecting the pollution status in the environment (Gouveia *et al.,* 2019).

In this paper, we developed a caging experiment where juvenile flounders collected in a "reference estuary" (the Canche) were c aged in the Canche, and in two polluted sites in the Seine estuary. The fish were exposed during one month to these environments, and their liver proteins were extracted and analyzed by shotgun proteomics. At the same time, flounders were sampled in Canche and Seine *in natura*, then compared with caged flounders in these sites, to assess the effect of caging on fish. The major aim of this study was to evaluate the relevance of a proteomic approach, for the discovery of key proteins produced by a fish often used as a sentinel species, the European flounder (Laroche et al., 2013), over an upstream-downstream gradient in a heavily polluted estuarine system.

Materials & Methods

Study sites

The study area was located along the French coast of the English Channel (Fig. 1). Two estuaries were investigated (Canche & Seine). The Canche is a small estuary with low freshwater input of 7 $m^3.s^{-1}$; it is located near a protected area and is not impacted by important human activities and is considered as a "pristine estuary" (Amara *et al.,* 2009). The Seine estuary is the largest estuary along the English Channel, concentrating the quarter of the French population, one third of the French industry and intensive agriculture (Mouny & Dauvin, 2002). The freshwater input varies from around 100-200 $m^3.s^{-1}$ in summer, to the maximum recorded in winter: 2,000 $m^3.s^{-1}$ (Mouny et al., 1998). In spite of significant efforts to restore the environmental quality of the Seine estuary during the past few decades, it remains one of the most chemically polluted estuaries in western northern Europe (Dauvin *et al*. 2007; Poisson, 2011).

Sampling and caging experiment

In September 2017, 150 0-group juvenile flounders *Platichthys flesus* (7–9 cm total length: TL) were collected in the Canche estuary by a small beam trawl; 30 juvenile flounders being also collected in the Seine estuary according to the same procedure (Fosse Nord site). A set of 30 fish per estuary were immediately analyzed after their catch in Canche and Seine; the objective was to compare the responses of flounders sampled in natura vs caged flounders. The remaining fish (*i.e.* 120 individuals from the Canche) were acclimatized for 1 week in a 500-L aquarium supplied with an open seawater circuit and were fed daily with frozen Mysidacea and brine shrimps (*Artemia* sp.). One day before the caging experiment, flounders were anesthetized (eugenol 35 mg/L), weighed (to the nearest 1 mg), measured for total length (within 0.1 mm), and individually marked (visual implant tag, 1.2 mm × 2.7

mm, Northwest Marine Technology). The VI Alpha Tags with an alphanumeric code designed to identify individual fish were implanted under the skin in the antero-dorsal part, using the VI Alpha Injector. The tags remained externally visible for easy recovery.

Cage settlement was carried out on the 12th and 13th of September 2017 at three different sites. Two sites were chosen in the Seine estuary: Rouen (49° 22.995' N; 01° 00.676' E), which is located near a wastewater treatment plant (Emeraude), and Fosse Nord (49° 27.328' N; 00° 07. 493' E), which is located in the main channel of the estuary (Fig. 1). The last caging site was located in the Canche estuary, close to the Etaples harbour (50° 30.982' N; 01° 37.852' E) (Fig. 1). Three cages were settled by site, each containing between 15 and 20 fish.

The cages were made of plastic. Their length was 1 m, whereas their width and height were 0.6 m. Their 15 mm mesh size facilitated water circulation. The cages were fixed to the bottom with two screw anchors secured by scuba divers, the average depth being 8 m and 3 m, respectively, in the Seine and Canche estuaries; these depths are representative of the highest densities of juvenile flounders in the two estuarine systems (Amara *et al*., 2009). After the one month caging, all fish were quickly transferred to the laboratory and then killed by cervical dislocation, the method classically used on juvenile flatfish (Dupuy *et al*., 2015); they were identified by their tags, weighed and measured. In order to evaluate the potential effect of contaminants on juvenile fish, we calculated the Fulton's condition index (K), considered as an indicator of the fish general well-being (Minier *et al.*, 2015). K was assessed with the formula : $k= 100*(W/L^3)$ where W= gutted body weight (i.e. carcass without internal organs) (g) and L = total length (cm) (Peig & Green, 2010). The same protocol was conducted on flounders trawled *in natura* (Canche and Seine), immediately after the catch. The liver and a blood sample were collected per fish, then flash-frozen in liquid nitrogen.

Pollutants analysis

Sample preparation and metals analysis in sediment

Dry sediment (200 mg) was totally digested in a heating block (HotBlock® SC100 Digestion System), with an acid mixture of aqua regia (HNO_3/HCl 1:3, v/v) and HF (10 mL) following a procedure described by Ouddane et al. (1999) with some modification. The sample was weighed into PTFE digestion cups, when the sediments were totally dissolved at 120 °C, the solution was then diluted with ultrapure Milli-Q+ water (18.2 MΩ cm) and filtered through a 0.45 µm cellulose acetate membrane. The metal concentrations were determined by inductively coupled plasma-atomic emission spectrometry ICP-AES (Agilent 5110, dual view) for the major element and by ICP-MS (Varian, 820) for the trace elements. Procedural blanks and certified reference materials (CRMs) MESS-3, HISS-1 and PACS-2 (National Research Council of Canada) were prepared using the same analytical procedure and reagents and analysed for quality assurance/quality control. All results were >92% confidence intervals.

For the fish samples (muscle tissues), the samples were homogenized and lyophilized. The samples were weighed into PTFE digestion cups and predigested at room temperature for 24 h with nitric acid, and then digested on a heating block (HotBlock® SC100 Digestion System) at a temperature of 120 °C. Digestion was performed until total decomposition (4-6 hours). The metal concentrations were determined by inductively coupled plasma-atomic emission spectrometry ICP-AES (Agilent 5110, dual view) for the major element and by ICP-MS (Varian, 820) for the trace elements. Reagent blanks were first run at intervals of every ten samples analysis to eliminate equipment drift. All samples were analysed in triplicates for reproducibility, accuracy and precision. Procedural blanks and certified reference materials (CRMs): DORM-3 (fish protein, National Research Council of Canada), and IAEA-436 (mussel tissue, International Atomic Energy Agency of Monaco), were analysed in the same conditions

as the samples, the results were in good agreement with the certified values, and the recoveries were within 10-15% of the certified values.

Organic pollutants in sediment

The concentrations of 24 HAPs, 26 PCBs, and 7 PBDEs were determined in sediments by stir bar sorptive extraction-thermal desorption-gas chromatography-tandem mass spectrometry (SBSE-GC-MS/MS) using a method adapted from Lacroix *et al*. (2014). Briefly, for each sample, 100 mg wet weight of sediment were digested by saponification and analytes were extracted for 16 hours at 700 rpm using polydimethylsiloxane stir-bars (Twister 20 mm x 0.5 mm, Gerstel). Bars were subsequently analysed using a gas chromatography system Agilent 7890A coupled to an Agilent 7000 triple quadripole mass spectrometer (Agilent Technologies) and equipped with a Thermal Desorption Unit (TDU) combined with a Cooled Injection System (Gerstel). The GC column was a Restek Rxi-5ms (30 m, 0.25 mm, 0.25 µm). Thermodesorption and GC-MS/MS conditions were as previously described. Analytes were quantified relatively to deuterated compounds using a calibration curve ranging from 0.01 ng to 30 ng per bar. A mean sediment water percentage of 81% was measured by drying samples at 50°C until the mass remained constant. Results are expressed as ng analytes/g dry weight (d.w.). Limits of quantification (LOQ) were calculated by the calibration curve method (Shrivastava and Gupta, 2011) and limit of detection (LOD) were estimated by dividing LOQ by 3. Analytical quality control was made using the Standard Reference Materials 1974c "Organics in Mussel Tissue *(Mytilus edulis)*" provided by the National Institute of Standards (SRM) and Technology (NIST, Gaithersburg, USA).

Genotoxicity

Blood samples diluted in cryopreservative buffer (1/100; 250 mM sucrose, 40 mM trisodium citrate, 5 % dimethyl sulfoxide, pH 7.6, adjusted with 1 M citric acid) were rapidly

thawed before the Comet assay, cell density was adjusted to 2,100 cells/µl of cold phosphate-buffered saline. Cell viability was checked by the Trypan blue exclusion method and was found to be >90 %. The Comet assay was performed on flounder erythrocytes according to the protocol described by Singh et al. (1988) with slight modifications. Resulting DNA was stained with Vista Green® Dye (OxySelectTM Comet Assay, Cliniscience), and analyzed with the CellInsight CX5 HCS® (Thermo). A total of 150 random cells on each slide were analyzed. % DNA Tail were measured for each cell.

Shotgun proteomics

Proteomic samples preparation

We chose to analyse the liver proteomes of fish encaged in the different estuaries because the liver is the main organ where detoxification metabolism occurs. Frozen livers obtained from 5 fish collected *in natura* (Canche) and 5 caged fish per site (Canche, Rouen, Fosse Nord) were each homogenized in 1 ml of Tris-HCl buffer (100 mM, pH 6.8) containing 1% Protease Inhibitor Mix (GE Healthcare), using a Precellys 24 system (Bertin Technologies). The supernatants were removed by centrifugation (12 000 g, 15 min, 4°C) and then incubated at ambient temperature for 30 min with 1% DNase mix (GE Healthcare). Total protein contents were determined using DC protein assay kit (biorad) in triplicate. A quantity of 30 µg of proteins was treated as previously described (Hartmann et al., 2014). Briefly, proteins were subjected to a short SDS-PAGE migration on a 4–12 % gradient 10-well NuPAGE gel (Invitrogen). The polyacrylamide bands corresponding to each whole proteome were excised. Proteins were first reduced with DTT and alkylated with iodoacetamide, and then proteolyzed with Trypsin Gold (Promega) in presence of 0.01 % ProteaseMAX surfactant (Promega) in 50mM NH_4HCO_3 for 1 h at 50 °C. Then, a volume of TFA 5 % was added to reach a final concentration of 0.5 %.

Q Exactive HF analysis

The cleaved peptides (5 µL injection containing 400 ng of acidified peptide lysate) were resolved by reverse phase chromatography on an Ultimate 3000 RSLC nano system (Thermo Scientific) over a 120-min linear gradient, 100 min from 4% B to 25% B and 20 min from 25% B to 40% B (A: 0.1% formic acid in MS-grade water; B: 80% acetonitrile, 0.1% formic acid in MS-grade water). Eluted peptides were online desalted and then analyzed by a Q Exactive HF mass spectrometer (Thermo Scientific) operated in data dependent mode with a Top20 strategy (= up to 20 MS/MS cycles after a full scan), with full scans at a resolution of 60,000 and most intense peaks with charge state 2 or 3 being selected for fragmentation (normalized collision energy at 27%) and subsequent MS/MS (resolution 15,000) measurements. Dynamic exclusion of previously measured precursor ion *m/z* values was set to 10s for improving spectral count results. The reverse phase column was then further eluted with 6 min of 90% B without recording MS/MS spectra, and equilibrated with 4% B.

Proteomic data interpretation

The recorded MS/MS spectra were searched with MASCOT against the Paralichthys olivaceus full annotated proteome database (Shao et al., 2017) which comprises 36,123 polypeptide sequences totaling 22,711,544 residues (GCF_001970005.1 v1.0, downloaded 2018-01-19). The following parameters were used: tryptic peptides with a maximum of 2 miscleavages during proteolytic digestion, a mass tolerance of 5 ppm on the parent ion and 0.02 Da on the MS/MS, fixed modification for carbamidomethylated Cys (+57.0215) and variable modification for oxidized Met (+15.9949) and deamidated Asn or Gln (+0.9840). All peptide matches with a peptide score above its query identity threshold set at $p < 0.05$ with the database and rank 1 were parsed using the IRMa 1.31.1c software (Dupierris et al., 2009). A protein was considered validated when at least two different peptides were detected. False-positive identification of proteins were estimated using a reverse decoy database as below

0.1% with these parameters.

The number of MS/MS spectra per protein (spectral counts) was determined for the five animals and the two technical replicates of each of the three sites. The protein abundances were compared for each Seine estuary sites to the Canche one. For each of these comparisons, the list of non-redundant proteins detected among the three corresponding datasets was established. The total spectral count of each polypeptide was used to rank the proteins from the highest to the lowest detection intensities. The statistical protein variations among the replicate samples of the three one-to-one comparisons were calculated using the T-Fold option of the PatternLab 2.0 software (Carvalho et al., 2008). This module allows normalizing the spectral count datasets, calculating the average fold changes with statistics (t-test), and estimating the resulting theoretical Benjamini-Hochberg false discovery rate. MS/MS data were compiled in Excel (Microsoft) and converted for PatternLab with an Excel home-designed macro. Normalization was done taking into account the total number of spectral count for each sample, taking at least two readings per protein. Parameters for the comparisons were as follows: minimum fold change of 1.5, minimum p-value of 0.05 and BH-FDR Alfa of 0.15.

For functional analyses, the sequences of the deregulated proteins were retrieved by using the “Batch Entrez module” available at www.ncbi.nlm.nih.gov. The four different subsets of proteins, corresponding to proteins up-and down-accumulated in fish encaged in the Rouen and Fosse Nord sites (as compared to fish encaged in Canche), as well as those deregulated following encagement in Canche (as compared to flounders fished in Canche), were then submitted to EggNOG 4.5.1 (Szklarczyk et al., 2016) and Blast2GO basic (Götz et al., 2008) for functional re-annotation, Gene Ontology (GO) and Cluster of Orthologous Group (COG) analyses. All results were carefully manually examined and corrected when necessary. For

KEGG enrichment analyses, protein sequences were submitted to STRING v11.0 (Szklarczyk et al., 2019), and analyzed against the genome of the closest available pleuronectiform fish annotated genome, ie. that of the tongue sole (*Cynoglossus semilaevis*). For these analyses, we kept a FDR threshold of significance of 10^{-5}.

Proteomic data deposition

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Perez-Riverol et al., 2019) with the dataset identifier PXD013684 (project DOI: 10.6019/PXD013684).

Results and discussion

Pollutants analysis

The Seine estuary is located downstream from the French largest urban area, and is known as one of the most polluted estuaries in France (Poisson, 2011). By contrast, the Canche estuary is located downstream of a protected natural reserve, and is most often considered a pristine ecosystem (Amara *et al.*, 2009; Kerambrun *et al.*, 2013). Our first objective was to characterize the actual pollution status of these ecosystems, by assessing the concentrations of several metals and organic pollutants in the three caging sites, two being located in the Seine estuary (Rouen, Fosse Nord), and one in the Canche estuary near the Etaples harbour (Fig. 1).

Analysis of the sediments collected in the three sites (Table 1) showed higher concentrations in Rouen *vs* Fosse Nord and Canche, for all of the measured metals (Ag, Cd, Hg, Hg, Mo, Co, Cu, Ni, Pb, V, Cr, Zn, Ba, Mn). The highest concentrations were observed for Manganese (533.1 $ng.g^{-1}$), Zinc (331.5 $ng.g^{-1}$), and Barium (264.2 $ng.g^{-1}$). Copper and Lead were also present in high concentrations at the Rouen site (100.1 $ng.g^{-1}$ and 93.0 $ng.g^{-1}$, respectively).

Surprisingly, most of the metals were more abundant in Canche vs Fosse Nord sediments (Table 1). Indeed, molybdenum, vanadium and manganese were two to three times higher levels in Canche; Zinc, Copper, Silver, Chromium and Cobalt displayed three to five times higher concentrations in Canche.

Globally, the lowest concentrations of organic pollutants were detected in the Canche estuary (Fig. 2). The values measured in the Canche sediments were 4,844 $ng.g^{-1}$ for PAHs, 96 $ng.g^{-1}$ for PCBs, and 3.6 $ng.g^{-1}$ for PBDEs. Expectedly, the Rouen site showed the highest concentrations of PAHs (7,418.6 $ng.g^{-1}$, almost twice those found in Canche) and PCBs (323.3 $ng.g^{-1}$, i.e. more than 3 times the concentration measured in Canche) (Fig. 2). The Fosse Nord sediments showed a PAHs level very close to that measured in the Canche (4,205.4 $ng.g^{-1}$), but higher concentration of PCBs (246.6 $ng.g^{-1}$). The highest concentration of PBDEs was detected in Fosse Nord, more than 4 times higher than in Canche (16.4 $ng.g^{-1}$ *vs* 3.6 $ng.g^{-1}$), intermediate concentrations being measured in Rouen (8.5 $ng.g^{-1}$) (Fig. 2). Globally, the analysis of metals and organic pollutants in the sediments confirmed that the Seine estuary is a highly polluted system, nevertheless displaying a decreasing pollution gradient from upstream to downstream; the chemical contamination of the Canche sediments being relatively moderate.

Phenotypic indicators

Flounders primarily fished in the Canche estuary were caged during one month in three sites: the Canche itself, and the Rouen and Fosse Nord sites located in the Seine estuary. A moderately higher condition index (K) was globally observed in fish caught *in natura vs* caged fish (Fig. 3). Considering only caged fish, a higher K was detected in Canche *vs* Fosse Nord, the difference with Rouen being insignificant (Fig. 3). The K variation is based on the assumption that heavier fish are in better condition and that higher K are generally observed in the least

contaminated station (Minier et al., 2015). Thus, in the present study the reduced K in caged vs in natura fish could be related to a stress induced by the caging, possibly linked to the limited availability of preys and/or the absence of shelter. For caged fish, the higher value of Canche K could be related to its moderate chemical stress; the reduced differences with the Seine polluted sites being probably explained by the short duration of the caging experiment (one month).

The muscle RNA/DNA ratios in caged fish, showed the higher value in the Rouen site, a significant difference being only detected between Rouen and Canche for fish *in natura*. A higher RNA/DNA ratio was clearly detected in Seine vs Canche (Fig. 3). Generally, higher RNA/DNA ratios are observed in fish in good condition and these ratios have been used to assess the effect of chemical pollution on fish (Amara *et al*., 2009; Minier *et al*., 2015). In the present work, the RNA/DNA ratio increased in the polluted sites *vs* the moderately contaminated sites, suggesting an increase of the flounder metabolic activities to cope with the chemical stress, as previously described in a study comparing the metabolism of juvenile flounders in Seine *vs* Canche (Dupuy *et al.*, 2015).

Numerous environmental pollutants were shown to behave as prooxidants that can produce an excess of reactive oxygen species, inducing different types of cellular alterations as lipid and protein oxidation and oxidative DNA damages; thus numerous biomarkers of genotoxicity were developed such as DNA breaks revealed by the Comet assay test (Amiard-Triquet and Berthet, 2015). Unsurprisingly, animals caged in the highly polluted site in the upper Seine (Rouen) showed the highest level of erythrocytes DNA damages (Fig. 3). Nevertheless, it should be noted that a similar high level of genotoxicity was also observed in the Canche caged fish; on the other hand, a reduced genotoxicity was observed in the Fosse Nord caged fish, as in the fish caught *in natura* in Seine and Canche (Fig. 3).

We suggest that the similar reduced genotoxicity observed in flounders caught *in natura* (Canche and Seine) is mainly due to a local adaptation of the Seine flounder population that have developed a tolerance to chronic exposure to high level of contaminants, reducing the impact of oxidative stress on DNA (Marchand et al., 2004). On the other hand, the introduction of "naive" flounders coming from Canche, in the cage located in the heavily polluted upstream Seine (Rouen), induced a significant increase of DNA damage.

The high level of genotoxicity also detected in the Canche caged fish was unexpected; it could be explained by the relatively high concentration of PAHs measured at the Canche site, close to the Etaples harbour, and/or by the influence of a confounding factor, i.e. a thermal stress inducing DNA damages. We hypothesize that the reduced cage depth in the Canche (average depth: 3 m) imposed by this shallow estuary could expose fish to a possible thermal stress during sunny conditions at the end of summer; heat stress creating DNA damages (Amiard-Triquet and Berthet, 2015). On the other hand, we suggest that the water temperature during the same season is probably more stable in the deeper cages of the Seine (average depth: 8 m) and thus could not be considered as a stressor for fish in this large estuary.

Shotgun proteomic approach

One of the most spectacular phenotypes in organisms subjected to adverse environmental conditions is a deep reprogramming of genetic and protein expression, that can be studied by global transcriptomic and proteomic approaches. In particular, proteomics can provide a better understanding of how organisms adapt their metabolism to counter the harmful effects of stress on cellular functions. Conversely, it can be assumed that the proteins expressed by an organism in a given ecosystem do reflect, to some extent, its environmental

conditions. Therefore, a global shotgun proteomic approach was developed which aimed at characterizing the proteins differentially expressed in the livers of *P. flesus* caught in the Canche estuary, but having undergone a 1-month encagement in the three estuarine sites covered by this study. For each condition, the proteomes of five animals were analyzed independently and technical duplicates were recorded for each animal.

In total, almost 3 million MS/MS spectra (2,980,656) were recorded, with an average of 74,516 MS/MS spectra per run. We analysed these spectra against the protein database of *Paralichthys olivaceus*, a flatfish species closely related to *P. flesus* with a fully sequenced genome (Shao *et al.,* 2015), and we could assign a total of 853,691 spectra to peptide sequences (Supplementary data, Table S1). On average, about 21,000 MS/MS spectra per run could be interpreted, i.e. an attribution rate of 28%, which is quite usual for a non-model organism and the use of a heterologous protein database. In total, no less than 3,761 proteins could be identified, including 2,511 certified by at least 2 different peptides (Table S2), on which the comparative statistical analysis was carried out using the PatternLab software. The analyses were carried out in pairs, each time using the fish caged in the Canche estuary as a reference, and applying as the thresholds of significance a p value of 0.05 and an amplification (or reduction) factor of 1.5 for protein abundance.

Proteomic signatures of the fish encaged in Rouen and Fosse Nord

The comparisons showed that the caged fish in the upstream Seine site (Rouen) accumulated 180 proteins, and down-accumulated 311 proteins, as compared to the fish encaged in the Canche estuary. Regarding the fish caged in the downstream Seine site (Fosse Nord), 82 proteins were found to have increased abundance and 458 were down-accumulated in this condition. Since Rouen and Fosse Nord are two sites in the same estuary, one could

have thought that part of the response would be common. In agreement, 31 proteins were found to be increased, and 131 proteins decreased in both sites. By contrast, very few proteins showed divergent results between the two sites; indeed, 8 were increased in Rouen and decreased in Fosse Nord, while 2 showed a reverse accumulation profile (decreased in Rouen and increased in Fosse Nord).

Functional analysis of deregulated proteins

Functional analysis makes it possible to visualize the main modifications and orientations of the metabolism under the different experimental conditions. To this end, the various protein subgroups were re-annotated using Blast2GO and EggNOG (Conesa *et al*., 2005; Powell *et al.,* 2014) softwares, followed by a careful manual re-examination. Results presented in Figure 4 show the number of proteins assigned to the different Clusters of Orthologous Group (COG) categories. They show that the main categories accumulated in the liver from fish caged in Rouen were related to the transport and metabolism of lipids (I), amino acids (E) and carbohydrates (G), as well as energy production and conversion (C). Concerning the Fosse Nord site, a single category clearly dominated the subgroups of accumulated proteins, *i.e.* the transport and metabolism of amino acids (E). It was followed by the categories I (lipid metabolism and transport) and C (energy production and conversion). For down-accumulated proteins (Fig. 4), the result were also very clear in fish caged in Fosse Nord, with three clearly dominant categories, i.e. translation, ribosomal structure and biogenesis (J), suggesting an overall depression of translational activity in cells, followed by categories O (PTM: post-translational modifications, protein turnover and chaperones) and U (intracellular trafficking), both related to protein processes and functioning. In the fish encaged in Rouen, the latter two categories were also affected, but the overall changes appeared more

diversified. Indeed, the category RNA processing and modification (A) seemed to be the most reduced, and categories T (signal transduction mechanisms) and Z (cytoskeleton) also appeared to be affected.

In order to go deeper in this functional analysis, we carried out KEGG enrichment analyses of all the deregulated protein subsets identified in this study, using the pleuronectiform flatfish genome *Cynoglossus semilaevis* as a reference. The results presented in Table 2 show a very significant enrichment of the pathways involved in the detoxification of drugs and xenobiotics (KEGG pathways 00982, 00980 and 00983) in the group of proteins accumulated by fish caged in Rouen. It is noteworthy that these pathways, which do not appear enriched in the livers of fish caged in Fosse Nord, seemed rather reduced in our study on the impact of caging, which only concerned fish caged (or not) in our control estuary (Canche). This analysis also suggested the stimulation of the peroxisomal lipids metabolism (csem04146) in the same condition (Rouen), as well as in fish caged in Fosse Nord, as compared to those encaged in Canche. For the rest, the overall results are very consistent with the previous COG analysis. These results are particularly interesting. Bearing in mind that the two polluted sites are in the same Seine estuary, but that the Rouen site has more metal and organic contamination (PCBs and PAHs), while the Fosse Nord site mainly contains PBDEs, we can expect common effects and different effects on fish caged at the two sites. Going deeper into the mechanisms, we can see more precisely how fish manage these different situations.

Deregulation of xenobiotic metabolism

As a rule, organisms react to the presence of xenobiotics by setting up excretion systems, and/or by activating metabolic systems, which are often non-specific or with very low specificity, aiming at increasing the water solubility of chemical compounds. The latter metabolic mechanisms are generally classified into Phase I and Phase II detoxification processes (Hassan *et al.*, 2015).

The Phase I mechanisms usually involve chemical reactions of hydrolysis, reduction and oxidation, exposing or introducing a functional group (-OH, -NH_2, -SH or -COOH) increasing the reactivity and hydrophilicity of the molecule. One major protein system involved in these reactions is represented by Mixed Function Oxidases (MFO), which require the coordinated action of a cytochrome P450 protein, together with a NADPH-Cytochrome P450 Reductase (Livingstone, 1998). These systems are often used to assess the ecotoxicological status of ecosystems, by measuring ethoxyresorufin-O-deethylase (EROD) activity, which is a recognized biomarker of the presence of pollutants such as PAHs, PCBs and dioxins for example (Porte *et al*., 2000; van der Oost *et al.*, 2003).

It is noteworthy that one NADPH-Cytochrome P450 Reductase is significantly positively deregulated in fish caged in Rouen, as well as five different isoforms of Cytochrome P450, which show induction factors that could reach 4.3 fold (Table 3). In fact, no less than ten different cytochrome P450 isoforms were found deregulated in our study. Five were specifically, and significantly, positively deregulated in Rouen, one was positively deregulated in fish from both Rouen and Fosse Nord sites, while four had their specific abundance significantly decreased in the Fosse Nord site, as compared to flounders encaged in the Canche estuary. Cytochromes P450 belong to a large family of enzymes, not only related to the metabolism of xenobiotics, but also to some normal cellular metabolisms such as biosynthesis and catabolism of sterols and steroid hormones, bile acids, fat-soluble vitamins, fatty acids and eicosanoids (Monostory and Dvorak, 2011). For example, thromboxane A synthase, which is also accumulated in Rouen fish, is also a P450 dependent enzyme involved in the metabolism of prostaglandin H2 (Hecker *et al*., 1987). Interestingly, only the four isoforms significantly positively deregulated in Rouen were assigned the GO term 0006805, "xenobiotic metabolic process". It is likely that only these four isoforms are actually related to high concentrations of

PAHs and PCBs (Van-Veld et al., 1997; Cresson et al., 2016), while the others, especially those negatively regulated in the Fosse Nord encaged fish, should rather be related to the general metabolism of the European flounder.

Other enzymes induced in fish from Rouen are also clearly associated with phase I reactions (Table 3). In particular, dimethylaniline monooxygenase is a NADPH dependent flavoprotein involved in the oxidation of many xenobiotics (Schlenk, 1998), drugs and pesticides, as well as epoxide hydrolase, which converts epoxide groups (often generated by MFOs) into diol (Peters et al., 1995); thus it is also an enzyme typically linked to phase I reactions (Buhler & Williams, 1988). Other oxidases such as amine oxidase and aldehyde oxidase that were found accumulated in Rouen fish livers could also catalyze Phase I reactions (Aker *et al*., 2008; Pryde *et al*., 2010). All of these proteins were assigned GO terms related to xenobiotic metabolism using Blast2GO.

We also observed a number of proteins related to Phase II reactions induced in fish encaged in the Seine estuary (Table 3). For example, three glutathione S-transferases were evidenced in our study, of which two were accumulated in the fish caged in Rouen, while the third one was accumulated in Fosse Nord fish. Like cytochrome P450, GSTs are an important family of proteins that play an essential role in the normal metabolism of cells, more specifically in cell redox homeostasis and adaptation to oxidative stress (Das *et al*., 2017). Only a few are directly involved in the detoxification of xenobiotics; they act by conjugating a glutathione molecule on xenobiotics, thus increasing their water solubility (Glisic *et al*., 2015).

Interestingly, in a recent proteomic study (Galland *et al*., 2015), we showed that *P. flesus* accumulated GST during an experimental exposure to a mixture of PAHs and PCBs reflecting the Seine contamination. This GST accumulation was related to the concomitant increase in many enzymes involved in the methionine cycle and the synthesis of cysteine, which

is a precursor of glutathione. Here again, we found some enzymes belonging to this cycle, such as an isoform of betaine homocysteine methyltransferase (BHMT) strongly induced in both Rouen and Fosse Nord fish, while two other isoforms of the same enzyme BHMT were down-accumulated in the fish encaged in Fosse Nord. Concomitantly, an S-adenosyl-methionine synthase was down-accumulated under both conditions. Interestingly, two other proteins related to this metabolism, i.e. cystathionine beta synthase, involved in the synthesis of cysteine, which is one of the precursors of glutathione, as well as adenosylhomocysteinase, which is important to feed the homocysteine pool in the cell, were accumulated in the Fosse Nord fish. Several works also conducted on the European flounders collected in the field (Dupuy et al., 2015), exposed to model toxicants (Williams *et al*., 2008) or to polluted estuarine sediments (Leaver at al., 2010) confirmed a significant induction of BHMT in all the polluted conditions. Thus, BHMT could then be considered as a robust stress marker for *P. flesus* and could be a link between classical responses to xenobiotics exposure such as detoxification, antioxidant responses, and methylation deregulations such as epigenetic DNA methylation (Galland *et al*., 2015).

Phase II metabolism involves conjugation reactions with glutathione, but also with other molecules such as glucuronic acid or sulfate. The addition of glucuronic acid to a xenobiotic molecule requires the enzyme UDP-glucuronosyl transferase (van der Oost et al., 2003). In our study, no less than four isoforms of this enzyme were found deregulated. Three were accumulated by fish encaged in Rouen, while one was found to be decreased in Fosse Nord. Interestingly, the latter was not assigned any GO term related to xenobiotics (unlike the others which were all assigned the GO:0006805 term, i.e. xenobiotic metabolic process), and could be linked to another metabolism. Regarding sulfatation mechanisms, only one protein was shown deregulated, but negatively, in the Fosse Nord site. This protein is a 3'-

phosphoadenosine 5'-phosphosulfate (PAPS) synthase, responsible for the activation of sulfate necessary for its conjugation (Klaassen & Boles, 1997). No sulfotransferase was observed. Nevertheless, the sulfotransferases involved in these Phase II reactions are generally known as non-inducible proteins (Stanley *et al*., 2005).

Beyond these Phase I and II reactions, which are relatively well documented, many other proteins could be involved in xenobiotic metabolism. Indeed, xenobiotics are by nature very diversified molecules that have been very recently introduced into natural ecosystems (as a result of industrialization). As a consequence, only very few cellular and molecular mechanisms are actually specific to a particular class of xenobiotics. Thus, the observed deregulation of many proteins associated with amino acids-, secondary metabolites- or lipids-metabolisms could either be a consequence, or an adaptations, to metabolize xenobiotics. For example, 3-oxo-5-beta-steroid 4-dehydrogenase (AKRD1D1) is an enzyme involved in steroid hormone metabolism (Cordeiro *et al*., 2012). It is questionable whether the increased abundance of this protein in fish caged in the Rouen site is a consequence of exposure to molecules resembling its substrates, or whether this change could generate endocrine disrupting effects. Cordeiro *et al*. (2012) suggested a plausible direct response of AKRD1D1 to higher levels of cortisol observed in chronically stressed fish (*Solea senegalensis*). Interestingly, authors highlighted a link between an immediate metabolite of cortisone catabolism generated by AKRD1D1 (the dihydrocortisone) and the BHMT overexpression. Specifically regarding endocrine disruption, we have not seen any usual markers such as vitellogenin or related proteins (not necessarily expected in an organ like the liver). Nevertheless, our results suggest that the liver cells of fish caged in polluted sites encounter major changes of intracellular signal transduction systems (Figure 4), especially at the Rouen site, which is likely to generate phenotypes related to endocrine perturbations.

General perturbation of the cell metabolism

A major modification pointed out by our study is related to the deregulation of carbon and energy metabolism in fish encaged in polluted sites. Examining more precisely the proteomic modifications in the fish encaged in Rouen, we observed an accumulation of the enzymes involved in the degradation of glycogen, but also of almost all the enzymes involved in the glycolytic pathway, and many enzymes involved in the tricarboxylic acid cycle, and in the mitochondrial electron transport chain. A large number of enzymes involved in the biosynthesis of fatty acids were also more abundant.

In the fish encaged at the Fosse Nord site, we did not find such deregulations. The metabolism even seems to go in the opposite direction regarding glycolysis and fatty acid synthesis. For example, the two Fatty Acid Synthase induced in Rouen were down-accumulated in Fosse Nord. On the other hand, as in Rouen, we observed a sharp decrease of several subunits of a V-type proton ATPase, a proton pump which is not involved in energy metabolism, but in the maintenance of pH homeostasis of organelles and more generally in the regulation of vesicular trafficking (Marshansky and Futai, 2008). This deregulation could be linked to the strong disturbance of the Golgi apparatus suggested by the many proteins assigned to the COG category "Intracellular trafficking, secretion, and vesicular transport", most of which being related to the functioning of the Golgi apparatus.

Another notable point is the impact on the defense mechanisms of fish encaged in Rouen and Fosse Nord, especially their immunity. Indeed, we observed in both sites a decrease in several complement proteins (1Q, C3, C5), but also in proteins linked to platelet aggregation (coagulation factor X, fibrinogen, fibronectin, antithrombin), which could have a deleterious effect on the ability of fish to defend themselves against possible pathogens. A convergent trend

was detected in a study on hepatic gene expression in flounder exposed to polluted estuarine sediments, where induction of inflammation and innate immune pathways were highlighted (Leaver *et al*., 2010).

Finally, the most significant point at Fosse Nord is linked to translation, as more than 100 proteins related to translation, ribosomal structure and biogenesis were down-accumulated, thus suggesting a general inhibition of the cell translational system. Similar response has already been described in response to a variety of stressing situations (Brostrom et al., 1996; von Born *et al*., 2018). In these situations, mRNAs are generally stored in specific structures, the so-called "stress granules", which include both poly(A)+ mRNA, ribosomal proteins (mainly 40S ribosomal subunits), eIF elongation factors, and PABP (polyadenylate binding protein, many elongation factors) (Mazroui *et al*, 2006; Anderson & Kedersha 2006). Most of these proteins were decreased in our study.

In the two polluted sites (Rouen and Fosse Nord), some stress markers, such as the HSP70 were decreased. Particularly in Fosse Nord, peroxiredoxin (an antioxidant enzyme) and proteins associated with proteasome function and more generally with stress-induced protein recycling (e.g. ubiquitin carboxyl-terminal hydrolase, proteasome subunits and proteasome regulatory subunits), were also significantly decreased. This result is quite difficult to interpret, because the presence of xenobiotics is known to cause stress at the cellular level, and most of these protein markers (HSPs, oxidative stress proteins and proteasome) are generally described as accumulated under such conditions (Kim & Kang, 2016; Wong & Do, 2017). As said previously, we wondered if the caging by itself could, for different reasons, induce a situation of significant stress for fish. As an attempt to test this hypothesis, we further analysed the proteomic modifications specifically induced by the encagement in Canche, by comparing the liver proteomes of the fish encaged in this “reference site”, to that of fish collected free in the

same environment.

Impact of encagement in the Canche on the liver proteome of the European flounder

The previous proteomic analysis showed important protein modifications in fish caged in the two polluted sites in the Seine estuary *vs* in the Canche estuary. In particular, we showed a positive deregulation of many proteins associated with the mechanisms of xenobiotics detoxification in fish caged in Rouen. Nevertheless, despite the chemical stress imposed by xenobiotics, some stress markers remained surprisingly down-accumulated, regardless of the polluted sites (Rouen or Fosse Nord). This result suggested that in our "control" condition (i.e. caging in the Canche estuary), the fish may have been subjected to environmental stressors (heat, salinity, hypoxia, etc.) of higher intensity than those encaged in the Seine estuary. This observation prompted us to question the impact of caging by itself on the proteomic phenotypes of fish. Therefore, we have also analyzed the hepatic proteomes of flounders that remained free, in the Canche, for the same period of the experiment (*in natura* condition), and compared them to the hepatic proteomes of fish encaged in the Canche, our reference estuary.

All fish came from the same population, and shared the same natural ecosystem, i.e. the Canche estuary, both before and during the caging period. One could therefore have expected minimal differences between the two conditions (encaged or not, in the same estuary). In fact, the results showed that some 605 proteins were significantly deregulated between the two conditions, which corresponded to 16% of the total observed proteome (3,774 proteins identified). Of these proteins, 367 were accumulated, and 238 showed a reduced abundance, in the livers of encaged fish as compared to the *in natura* condition (Table S2). Fish physiology seems therefore to be deeply modified in this situation of encagement.

We first performed a global comparison of the three sets of proteins deregulated in fish encaged in Rouen and Fosse Nord and those that had remained free in the Canche (*in natura* condition), as compared to those caged in the Canche estuary. This enabled to show many proteins commonly deregulated in these three conditions, most of these proteins showing a similar trend (Fig. 5). In particular, 34 proteins were found to accumulate, and 110 were decreased in the three conditions. This suggests that the deregulation of these proteins is linked to the particular environmental conditions encountered when caging in the Canche, rather than the particular conditions found in the other sites.

Having a deeper look at the results (Table S2), it seems that the carbon metabolism and energy production were severely disrupted in encaged fish. Indeed, many proteins involved in the degradation of glycogen, in the glycolysis pathway, in the Krebs cycle, but also in the mitochondrial electron transport chain were down-accumulated during caging. Lipid synthesis, i.e. both the elongation of fatty acids and the metabolism of sterols, were also reduced. The caging procedure therefore seems to lead to a slowdown in energy metabolism, which is most probably related to the drop in fitness suggested by the 8.5% decrease in the condition factor of encaged flounders, as compared to the *in natura* condition (Fig. 3).

In addition to the carbon and energy metabolism proteins evoked above, the abundance of many proteins revealed modified. Performing functional analyses following the same procedure as for the inter-estuary comparison, we determined that the dominant COG category in the set of proteins accumulated in the caging situation was "RNA processing and modification" (56 proteins), to which we could add 22 proteins involved in the translation process. Many proteins were involved in signal transduction (38 accumulated and 17 reduced) and post-translational modifications (41 induced and 12 reduced). These proteins contribute to the alternative regulation of cellular functioning which would therefore mainly take place at

the post-transcriptional level, under caging conditions.

Regarding the metabolism of xenobiotics, we observed a significant enrichment in KEGG pathways involved in both Phase I and Phase II reactions in the subset of proteins which amount decreased during caging (ie. csem00980, 00982, 00983, 00480, Table 2). Indeed, no less than 9 Cytochromes P450, NADPH cytochrome P450 reductase, dimethylaniline monooxygenase, epoxide hydrolase, 5 glutathione S-transferases and 3 UDP-glucuronosyl transferases were reduced in abundance in the encaged fish. This was also the case for betaine homocysteine methyl transferase and serine hydroxymethyl transferase, two proteins involved in the methionine cycle previously found to be related to xenobiotic adaptation in previous ecotoxicoproteomics studies we performed to investigate the impact of pollution exposure in the Seine estuary (Galland et al., 2013, 2015).

Regarding the stress proteins, we observed the accumulation during caging of chaperones (2 HSP70, 2 DnaJ), oxidative stress proteins (2 thioredoxins, peroxyredoxin), as well as of proteins belonging to the proteins recycling system linked to the response to cellular stress (many components of the proteasome, 4 ubiquitin carboxy-terminal hydrolases), and to a protein known to be positively regulated in hypoxic situations (Hypoxia upregulated protein). Interestingly, most of these proteins are commonly down-accumulated in the three comparisons we made (Fig. 5), thus suggesting that the specific environmental conditions encountered by fish during caging in Canche created a stressful situation (more than the caging by itself). In particular, the shallow depth of the submerged cages in Canche due to the reduced bathymetry of this small estuarine system, and to the proximity of these cages to the marina, could have constituted sources of stress for the fish caged in this estuary.

Concluding remarks

In this study, we collected European flounder juveniles from the Canche estuary, an ecosystem close to a protected area known to be essentially free of pollutants. These fish were caged for one month in the Canche estuary, used as the control, and in two different sites in the Seine estuary showing contrasted levels of pollution. Determining pollutants in the sediments of the three sites, we showed very high contamination levels of metals, PAHs and PCBs in the Rouen site, whereas the Fosse Nord site was mainly polluted by PBDEs. The proteomic phenotypes of fish caged in the polluted sites, as compared to those caged in the Canche estuary, revealed major changes in the general cellular functioning, including e.g. increase in energy metabolism and fatty acid synthesis in Rouen, a general depletion of the translational system, more strongly marked at the Fosse Nord site, and an overall impact on signal transduction processes and intracellular traffic processes, that should be related to a general malfunction of the Golgi apparatus. Beyond these general changes, we have seen an increase in many proteins clearly associated with the detoxification mechanisms of xenobiotics, both Phase I and Phase II mechanisms. The increase in these proteins was mainly observed in the fish encaged in Rouen, in strong agreement with the pollution data we acquired for this site.

The caging technique allows transplanting a homogeneous population of fish into different environments, thus reducing the risks of bias due to their genetic variability, while retaining most of the complexity of the natural environment; as such, it is a very attractive technique in an environmental assessment context. Thus, caging techniques have been used widely for field exposures of fish to chemical contaminants, but only rare studies have evaluated the physiological consequence of caging fish (Oikari, 2006). In order to better interpret the previous data, it was thus important to evaluate the impact of caging on the

overall physiology of these fish. It should be kept in mind, for interpretation, that our results only considered two polluted- and one control- sites, and the effects of encagement in only the last one. However, the present study strongly suggested that the comparison of fish in cages to their free-living counterparts is an appropriate way to evaluate the status of fish used as references. Our proteomic approach clearly revealed the accumulation of contamination protein markers in the livers of fish encaged in the Seine polluted sites, but it also underlined a more intense exposure of Canche caged fish to physico-chemical stressors, especially heat stress. Therefore, our study emphasized the fact that in complex estuarine ecosystems, numerous environmental parameters could become stressors for caged fish such as limited access to nutritional resources, and physico-chemical parameters of the micro-habitat around the cage, which do not necessarily represent the overall heterogeneity of the estuarine environment.

Acknowledgements

This work was supported by the “Région Bretagne” and the projects: Seine Aval 6 – HQFish and Agence Française pour la Biodiversité – POPEST.

References

Aker, W.G., Hu, X., Wang, P., Hwang, H. (2008). Comparing the relative toxicity of malathion and malaoxon in blue catfish *Ictalurus furcatus.* Environ. Toxicol. 23(4), 548-554.

Anderson, P., Kedersha, N. (2006). RNA granules. J Cell Biol, 172(6), 803-808.

Amara, R., Méziane, T., Gilliers, C., Hermel, G., Laffargue, P. (2007). Growth and condition indices in juvenile sole *Solea solea* measured to assess the quality of essential fish habitat. Mar. Ecol. Prog. Ser. 351, 201-208.

Amara, R., Selleslagh, J., Billon, G., & Minier, C. (2009). Growth and condition of 0-group European flounder, *Platichthys flesus* as indicator of estuarine habitat quality. Hydrobiologia. 627(1), 87-98.

Amiard-Triquet, C., Berthet, B. (2015). Individual biomarkers. In Amiard-Triquet, C., Amiard, J.C., Mouneyrac, C. Aquatic Ecotoxicology: advancing tools for dealing with emerging risks. Academic Press. 153-182.

Archambault, B., Rivot, E., Savina, M., Le Pape, O. (2015). Using a spatially structured life cycle model to assess the influence of multiple stressors on an exploited coastal-nursery-dependent population. Estuar. Coast. Shelf Sci. 201, 95-104.

Aubry, A., Elliott, M. (2006). The use of environmental integrative indicators to assess seabed disturbance in estuaries and coasts: application to the Humber Estuary, UK. Mar. Pollut. Bull. 53(1-4), 175-185.

Beck, M. W., Heck, K. L., Able, K.W., Childers, D. L., Eggleston, D. B., Gillanders, B. M., Halpern, B., Hays, C.G., Hoshino, K., Minello, T.J., Orth, R.J., Sheridan, P.F., Weinstein, M.P. (2001). The identification, conservation, and management of estuarine and marine nurseries for fish and invertebrates: a better understanding of the habitats that serve as nurseries for marine species and the factors that create site-specific variability in nursery quality will improve conservation and management of these areas. AIBS Bull., 51(8), 633-641.

Blanchet, H., Lavesque, N., Ruellet, T., Dauvin, J. C., Sauriau, P. G., Desroy, N., Desclaux, C., Leconte, M., Bachelet, G., Janson, A.-L., Bessineton, C., Duhamel, S., Jourde, J., Mayot, S., Simon, S., de Montaudouin, X. (2008). Use of biotic indices in semi-enclosed coastal ecosystems and transitional waters habitats—implications for the implementation of the European Water Framework Directive. Ecol. Indic. 8(4), 360-372.

Borcier, E., Charrier, G., Amérand, A., Théron, M., Loizeau, V., Pédron, N., Laroche, J. (2016). Bioenergetic transcriptomic responses of European flounder (*Platichthys flesus)* populations in contrasted environments: impacts of pollution and global warming. JoX. 6(2).

Brostrom, C. O., Prostko, C. R., Kaufman, R. J., Brostrom, M. A. (1996). Inhibition of Translational Initiation by Activators of the Glucose-regulated Stress Protein and Heat Shock Protein Stress Response Systems. J. Biol. Chem. 271(40), 24995-25002.

Buhler, D.R., Williams, D.E. (1988). The role of biotransformation in the toxicity of chemicals. Aquat. Toxicol. 11, 19-28.

Burgeot, T., Akcha, F., Menard, D., Robinson, C., Loizeau, V., Brach-Papa, C., Martinez-Gomez, C., Le Goff, J., Budzinski, H., Le Menach, K., Cachot, J., Minier, C., Broeg, K., Hylland, K. (2017). Integrated monitoring of chemicals and their effects on four sentinel species, *Limanda limanda*, *Platichthys flesus*, *Nucella lapillus* and *Mytilus* sp., in Seine Bay: A key step towards applying biological effects to monitoring. Mar. Environ. Res. 124, 92-105.

Cachot, J., Geffard, O., Augagneur, S., Lacroix, S., Le Menach, K., Peluhet, L., Couteau, J., Denier, X., Devier, M.H., Pottier, D., Budzinski, H. (2006). Evidence of genotoxicity related to high PAH content of sediments in the upper part of the Seine estuary (Normandy, France). Aquat. Toxicol. 79(3), 257-267.

Carpentier, S., Moilleron, R., Beltran, C., Herve, D., Thevenot, D. (2002). Quality of dredged material in the river Seine basin (France). II. Micropollutants. Sci. Tot. Environ. 299(1-3), 57-72.

Carvalho, P.C., Fischer, J.S., Chen, E.I., Yates, J.R., & Barbosa, V.C. (2008). PatternLab for proteomics: a tool for differential shotgun proteomics. BMC Bioinformatics. 9(1), 316.

Chapman, P. M., Wang, F. (2001). Assessing sediment contamination in estuaries. Environ. Toxicol. Chem. 20, 3-22.

Conesa, A., Götz, S., García-Gómez, J. M., Terol, J., Talón, M., Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics, 21(18), 3674-3676.

Cordeiro, O.D., Silva, T.S., Alves, R.N., Costas, B., Wulff, T., Richard, N., de Vareilles, M., Conceição, L.E.C., Rodrigues, P.M. (2012). Changes in liver proteome expression of Senegalese sole (*Solea senegalensis*) in response to repeated handling stress. 14(6), 714-729.

Costanza, R., d'Arge, R., deGroot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., Oneill, R.V., Paruelo, J., Raskin, R.G., Sutton, P., VandenBelt, M. (1997). The value of the world's ecosystem services and natural capital. Nature. 387, 253-260.

Courrat, A., Lobry, J., Nicolas, D., Laffargue, P., Amara, R., Lepage, M., Girardin, M., Le Pape, O. (2009). Anthropogenic disturbance on nursery function of estuarine areas for marine species. Estuar. Coast. Shelf S. 81(2), 179-190.

Cresson, P., Fabri, M.C., Marco-Miralles, F., Dufour, J.L., Elleboode, R., Sevin, K., Mahe, K., Bouchoucha, M. (2016). Variability of PCB burden in 5 fish and sharks species of the French Mediterranean continental slope. Environ. Pollut. 212, 374-381.

Das, D., Moniruzzaman, M., Sarbajna, A., Chakraborty, S.B. (2017). Effect of heavy metals on tissue – specific antioxidant response in indian major carps. Environ. Sci. Pollut. Res. 24(22), 18010-18024.

Dauvin, J.C., Ruellet, T., Desroy, N., Janson, A.L. (2007). The ecological quality status of the Bay of Seine and the Seine estuary: use of biotic indices. Mar. Pollut. Bull. 55(1-6), 241-257.

Dauvin, J.C., Ruellet, T. (2009). The estuarine quality paradox: is it possible to define an ecological quality status for specific modified and naturally stressed estuarine ecosystems ? Mar. Pollut. Bull. 59(1-3), 38-47.

Dauvin, J.C. (2018). Twenty years of application of Polychaete/Amphipod ratios to assess diverse human pressures in estuarine and coastal marine environments: A review. Ecol. Indic. 95, 427-435.

Dévier, M.H., Le Dû-Lacoste, M., Akcha, F., Morin, B., Peluhet, L., Le Menach, K., Burgeot, T., Budzinski, H. (2013). Biliary PAH metabolites, EROD activity and DNA damage in dab (*Limanda limanda*) from Seine Estuary (France). Environ. Sci. Pollut. Res. 20(2), 708-722.

Dupierris, V., Masselon, C., Court, M., Kieffer-Jaquinod, S., Bruley, C. (2009). A toolbox for validation of mass spectrometry peptides identification and generation of database: IRMa. Bioinformatics, 25(15), 1980-1981.

Dupuy, C., Galland, C., Pichereau, V., Sanchez, W., Riso, R., Labonne, M., Amara, R., Charrier, G., Fournier, M., Laroche, J., 2015. Assessment of the European flounder responses to chemical stress in the English Channel, considering biomarkers and life history traits. Mar. Pollut. Bull. 95(2), 634-645.

Edgar, G.J., Barrett, N.S., Graddon, D. J., Last, P.R. (2000). The conservation significance of estuaries: a classification of Tasmanian estuaries using ecological, physical and demographic attributes as a case study. Biol. Conserv. 92(3), 383-397.

Erraud, A., Bonnard, M., Geffard, O., Chaumot, A., Duflot, A., Geffard, A., Forget-Leray, J., Xuereb, B. (2018). Assessment of sperm DNA integrity within the *Palaemon longirostris* (H.) population of the Seine estuary. Environ. Pollut. 245, 485-493.

Galland, C., Dupuy, C., Capitaine, C., Auffret, M., Quiniou, L., Laroche, J., Pichereau, V. (2013). Comparisons of liver proteomes in the European flounder *Platichthys flesus* from three contrasted estuaries. J. Sea Res. 75, 135-141.

Galland, C., Dupuy, C., Loizeau, V., Danion, M., Auffret, M., Quiniou, L., Laroche, J., Pichereau, V. (2015). Proteomic analysis of the European flounder *Platichthys flesus* response to experimental PAH–PCB contamination. Mar. Pollut. Bull. 95(2), 646-657.

Glisic, B., Mihaljevic, I., Popovic, M., Zaja, R., Lonca, J., Fent, K., Kovacevic, R., Smital, T. (2015) Characterization of gluthathione - S - transferases in zebrafish (*Danio rerio*). Aquat. Toxicol. 158, 50-62.

Gouveia, D., Almunia, C., Cogne, Y., Pible, O., Degli-Esposti, D., Salvador, A., Cristobal, S., Sheehan, D., Chamot, A., Geffard, O., Armengaud, J. (2019). Ecotoxicoproteomics: A decade of progress in our understanding of anthropogenic impact on the environment. J. Proteomics. 198, 66-77.

Götz, S., García-Gómez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., Robles, M., Talon, M., Dopazo, J., Conesa, A. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic acids res. 36(10), 3420-3435.

Hartmann, E.M., Allain, F., Gaillard, J.C., Pible, O., Armengaud, J. (2014). Taking the shortcut for high-throughput shotgun proteomic analysis of bacteria. Methods Mol. Biol. 1197:275-285.

Hassan, I., Jabir, N.R., Ahmad, S., Shah, A., Tabrez, S. (2015). Certain phase I and II enzymes as toxicity biomarkers: an overview. Water Air Soil Pollut. 226, 153.

Hecker, M., Baader, W.J., Weber, P., Ullrich, V. (1987). Thromboxane synthase catalyses

hydroxylations of prostaglandin H_2 analogs in the presence of iodosylbenzene. Eur. J. Biochem. 169: 563-569.

Kerambrun, E., Henry, F., Cornille, V., Courcot, L., Amara, R., 2013. A combined measurement of bioaccumulation and condition indices in juvenile European flounders, *Platichthys flesus*, from European estuaries. Chemosphere. 91, 498-505.

Kim, J.H., Kang, J.C. (2016). The toxic effects on the stress and immune responses in juvenile rockfish, *Sebastes schlegelii* exposed to hexavalent chromium. Environ. Toxicol. Pharmacol. 43, 128-133.

Klaassen, C.D., Boles, J.W. (1997). Sulfation and sulfotransferases 5: the importance of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) in the regulation of sulfation. FASEB J. 11, 404-418.

Lacroix, C., Le Cuff, N., Receveur, J., Moraga, D., Auffret, M., Guyomarch, J. (2014). Development of an innovative and green stir bar sorptive extraction–thermal desorption–gas chromatography–tandem mass spectrometry method for quantification of polycyclic aromatic hydrocarbons in marine biota. J. Chromatogr. A. 1349, 1–10.

Laroche, J., Gauthier, O., Quiniou, L., Devaux, A., Bony S., Evrard, E., Cachot, J., Chérel, Y., Larcher, T., Riso, R., Pichereau, V., Devier, M.H., Budzinski, H. (2013). Variation patterns in individual fish responses to chemical stress among estuaries, seasons and genders: the case of the European flounder (*Platichthys flesus*) in the Bay of Biscay. Environ. Sci. Pollut. Res. Int. 20, 738-748.

Leaver, M., Diab., A., Boukouvala, E., Williams, T.D., Chipman, J.K., Moffat, C.F., Robinson, C.D., George, S.G. (2010). Hepatic gene expression in flounder chronically exposed to multiply polluted estuarine sediment: absence of classical exposure "biomarker" signals and induction of inflammatory, innate immune and apoptotic pathways. Aquat. Toxicol. 96, 234-245.

Le Pape, O., Gilliers, C., Riou, P., Morin, J., Amara, R., Désaunay, Y. (2007). Convergent signs of degradation in both the capacity and the quality of an essential fish habitat: state of the Seine estuary (France) flatfish nurseries. Hydrobiologia. 588, 225-229.

Livingstone, D.R. (1998). The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 120, 43-49.

Lotze, H.K., Lenihan, H.S., Bourque, B.J., Bradbury, R.H., Cooke, R.G., Kay, M.C., Kidwell, S.M., Kirby, M.X., Peterson, C.H., Jackson, J.B. (2006). Depletion, degradation, and recovery potential of estuaries and coastal seas. Science. 312, 1806-1809.

Marchand, J., Quiniou, L., Riso, R., Thebaut, M.T., Laroche, J. (2004). Physiological cost of tolerance to toxicants in the European flounder *Platichthys flesus*, along the French Atlantic Coast. Aquat. Toxicol. 70(4), 327-343.

Marshansky, V., Futai, M. (2008). The V-type H^+-ATPase in vesicular trafficking: targeting, regulation and function. Current Opinion in Cell Biology. 20(4): 415-426.

Martens, L., Hermjakob, H., Taylor, C., Gevaert, K., l Vandekerckhove, J., & Apweiler, R. (2004). The PRIDE Database: Giving Researchers Access to Proteomics Data and Providing This Data with a

Home. Mol. Cell. Proteomics. 3, S257.

Mazroui, R., Sukarieh, R., Bordeleau, M. E., Kaufman, R.J., Northcote, P., Tanaka, J., ... & Pelletier, J. (2006). Inhibition of ribosome recruitment induces stress granule formation independently of eukaryotic initiation factor 2α phosphorylation. Mol. Biol. Cell. 17, 4212-4219.

Minier, C., Amara, R. (2008). From pollution to alterated fish physiological performance; the case of flatfish in the Seine estuary. In Amiard-Triquet, C. and Rainbow, P.S. Environmental assessment of estuarine ecosystems: a case study. Taylor and Francis. Boca Raton. 227-240.

Minier, C., Amara, R., Lepage, M. (2015). Fish as reference species in different water masses. In Amiard-Triquet, C., Amiard, J.C., Mouneyrac, C. Aquatic Ecotoxicology: advancing tools for dealing with emerging risks. Academic Press. 309-331.

Minier, C., Levy, F., Rabel, D., Bocquené, G., Godefroy, D., Burgeot, T., Leboulenger, F. (2000). Flounder health status in the Seine Bay. Mar. Env. Res. 50, 373-377.

Monostory, K., Dvorak, Z. (2011). Steroid regulation of drug-metabolizing P450. Curr. Drug. Metab. 12, 154-172.

Mouny, P., Dauvin, J. C., Bessineton, C., Elkaim, B., Simon, S. (1998). Biological components from the Seine estuary: first results. In Oceans, Rivers and Lakes: Energy and Substance Transfers at Interfaces (pp. 333-347). Springer, Dordrecht.

Mouny, P., Dauvin, J.C. (2002). Environmental control of mesozooplankton community structure in the Seine estuary (English Channel). Oceanol. Acta. 25, 13-22.

Ohe, T., Watanabe, T., Wakabayashi, K. (2004). Mutagens in surface waters: a review. Mutat. Res. 567, 109-149.

Oikari, A. (2006). Caging techniques for field exposures of fish to chemical contaminants. Aquat. Toxicol. 78, 370-381.

Ouddane, B., Skiker, M., Fischer, J.C., Wartel, M. (1999). Distribution of iron and manganese in the Seine river estuary: approach with experimental laboratory mixing. J. Environ. Monit. 1, 489-496.

Pedron, N., Artigaud, S., Zambonino-Infante, J.L., Le Bayon, N., Charrier, G., Pichereau, V., Laroche, J. (2017). Proteomic responses of the European flounder to multiple stressors: differential sensitivities of peripheral vs core populations. Sci. Tot. Environ. 586, 890-899.

Peig, J., Green, A.J. (2010). The paradigm of body condition: a critical reappraisal of current methods based on mass and length. Funct. Ecol. 24(6), 1323-1332.

Perez-Riverol, Y., Csordas, A., Bai, J., Bernal-Llinares, M., Hewapathirana, S., Kundu, D.J., Inuganti, A., Griss, J., Mayer, G., Eisenacher, M., Pérez, E., Uszkoreit, J., Pfeuffer, J., Sachsenberg, T., Yilmaz, S., Tiwary, S., Cox, J., Audain, E., Walzer, M., Jarnuczak, A.F., Ternent, T., Brazma, A., Vizcaíno, J.A. (2019). The PRIDE database and related tools and resources in 2019: improving support for quantification data. Nucleic Acids Res. 47(D1):D442-D450.

Peters, L.D., Livingstone, D.R., Shenin-Johnson, S., Hines, R.N., Schlenk, D. (1995). Characterization of hepatic Flavin monooxygenase from the marine teleost turbot (*Scophthalmus maximus* L.).

Xenobiotica. 25(2): 121-131.

Peterson, C.H., Jackson, J.B. (2006). Depletion, degradation, and recovery potential of estuaries and coastal seas. Science. 312(5781), 1806-1809.

Poisson, E. (2011). Effets de la contamination chimique: des organismes en danger ? Fascicule Seine-Aval 2, Groupement d'Intérêt Publique Seine-Aval. 1-69.

Porte, C., Escartín, E., García, L.M., Solé, M., Albaigés, J. (2000). Xenobiotic metabolising enzymes and antioxydant defences in deep-sea fish : relationship with contaminant body burden. Mar. Ecol. Prog. Ser. 192, 259-266.

Powell, S., Forslund, K., Szklarczyk, D., Trachana, K., Roth, A., Huerta-Cepas, J., Gabaldon, T., Rattei, T., Creevey, C., Kuhn, M., Jensen, L.J., Von Mering, C., Bork, P. (2014). eggNOG v4. 0: nested orthology inference across 3686 organisms. Nucleic acids research, 42, D231-D239.

Pryde, D.C., Dalvie, D., Hu, Q., Jones, P., Obach, R.S., Tran, T. (2010). Aldehyde oxidase: an enzyme of emerging importance in drug discovery. J. Med. Chem. 53(24): 8441-8460.

Riou, P., Le Pape, O., Rogers, S.I. (2001). Relative contributions of different sole and plaice nurseries to the adult population in the Eastern Channel: application of a combined method using generalized linear models and a geographic information system. Aquat. Living Resour. 14, 125-135.

Rochette, S., Rivot, E., Morin, J., Mackinson, S., Riou, P., Le Pape, O. (2010). Effect of nursery habitat degradation on flatfish population: Application to *Solea solea in* the Eastern Channel (Western Europe). J. Sea Res. 64, 34-44.

Schlenk, D. (1998). Occurrence of flavin-containing monooxygenases in non-mammalian eukaryotic organisms. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 121, 185-195.

Schloesser, R. W., & Fabrizio, M. C. (2018). Nursery habitat quality assessed by the condition of juvenile fishes: not all estuarine areas are equal. Estuaries and Coasts. 42, 548-566.

Shao, C., Niu, Y., Rastas, P., Liu, Y., Xie, Z., Li, H., Wang, L., Jiang, Y., Tai, S., Tian, Y., Sakamoto, T., Chen, S. (2015). Genome-wide SNP identification for the construction of a high-resolution genetic map of Japanese flounder (*Paralichthys olivaceus*): applications to QTL mapping of *Vibrio anguillarum* disease resistance and comparative genomic analysis. DNA Res. 22(2), 161-170.

Shao, C., Bao, B., Xie, Z., Chen, X., Li, B., Jia, X., ... Hamre, K. (2017). The genome and transcriptome of Japanese flounder provide insights into flatfish asymmetry. Nat. Genet. 49(1), 119. doi: 10.1038/ng.3732.

Shrivastava, A. and Gupta, V. B., 2011. Methods for the determination of limit of detection and limit of quantification of the analytical methods. Chron. Young Sci. 2, 21–25.

Singh, N. P., McCoy, M. T., Tice, R. R., Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell. Res. 175(1), 184-191.

Stanley, E. L., Hume, R., Coughtrie, M.W. (2005). Expression profiling of human fetal cytosolic sulfotransferases involved in steroid and thyroid hormone metabolism and in detoxification.

Mol. Cell. Endocrinol. 240(1-2), 32-42.

Szklarczyk, D., Morris, J. H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., Santos, A., Doncheva, N.T., Roth, A., Bork., P., Jensen, L.J., von Mering, C. (2016). The STRING database in 2017: quality-controlled protein–protein association networks, made broadly accessible. Nucleic Acids Res. 45, 362-368.

Szklarczyk, D., Gable, A.L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M., Doncheva, N.T., Morris, J.H., Bork, P., Jensen, L.J., von Mering, C. (2019). STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. 47, D607-613.

Tecchio, S., Chaalali, A., Raoux, A., Rius, A. T., Lequesne, J., Girardin, V., Lassalle, G., Cachera, M., Riou, P., Lobry, J., Dauvin, J.C., Niquil, N. (2016). Evaluating ecosystem-level anthropogenic impacts in a stressed transitional environment: The case of the Seine estuary. Ecol. Indic., 61, 833-845.

Tice, R. R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y. F. (2000). Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ. Mol. Mutagen. 35(3), 206-221.

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

Van Veld, P.A., Vogelbein, W.K., Cochran, M.K., Goksoyr, A., Stegeman, J.J. (1997). Route-specific patterns of cytochrome P450 1A (CYP1A) expression in fish (*Fundulus heteroclitus*) following exposure to aqueous and dietary benzo[a]pyrene. Toxicol. Appl. Pharmacol., 142, 348-359.

von Born, P., Bernardo-Faura, M., Rubio-Somoza, I. (2018). An artificial miRNA system reveals that relative contribution of translational inhibition to miRNA-mediated regulation depends on environmental and developmental factors in *Arabidopsis thaliana*. PLOS One. 13(2), e0192984.

Williams, T.D., Diab, A., Ortega, F., Sabine, V.S., Godfrey, R.E., Falciani, F., Chipman, J.K., George, S.G. (2008). Transcriptomic responses of European flounder (P*latichthys flesus*) to model toxicants. Aquat. Toxicol. 90, 83-91.

Wong, L.L., Do, D. T. (2017). The Role of Heat Shock Proteins in Response to Extracellular Stress in Aquatic Organisms. In Heat Shock Proteins in Veterinary Medicine and Sciences (pp. 247-274). Springer, Cham.

Table 1: Metal concentrations (in $ng.g^{-1}$) in sediment, for the three caging sites

	Ag	Ba	Cd	Co	Cr	Cu	Hg	Mn	Mo	Ni	Pb	V	Zn
Canche	0.34	142.5	0.37	4.43	27.73	6.77	0.34	169.53	0.34	7.62	11.59	26.88	38.26
Fosse Nord	0.12	127.5	0.38	1.55	6.77	2.34	0.27	83.30	0.23	8.59	9.30	10.68	11.26
Rouen	7.13	264.26	2.76	12.06	79.23	100.14	0.65	533.14	0.99	37.7	93.02	63.67	331.54

Table 2: KEGG enrichment analyses of the different subsets of proteins found deregulated in this study*.

Proteins subset	KEGG pathway	description	count in gene set	FDR
Rouen up	csem01100	Metabolic pathways	69 of 1433	1.65e-33
	csem04146	Peroxisome	16 of 91	1.13e-14
	csem00982	Drug metabolism - cytochrome P450	8 of 28	6.94e-09
	csem00980	Metabolism of xenobiotics by cytochrome P450	8 of 32	1.37e-08
	csem00260	Glycine, serine and threonine metabolism	8 of 47	1.64e-07
	csem00983	Drug metabolism - other enzymes	8 of 57	5.44e-07
	csem00500	Starch and sucrose metabolism	7 of 40	8.80e-07
	csem00380	Tryptophan metabolism	7 of 46	1.84e-06
	csem00010	Glycolysis / Gluconeogenesis	8 of 82	4.95e-06
Rouen down	csem03040	Spliceosome	27 of 142	6.74e-19
	csem04145	Phagosome	17 of 157	2.05e-08
	csem04142	Lysosome	16 of 158	1.10e-07
	csem04144	Endocytosis	19 of 325	1.09e-05
Fosse Nord up	csem01100	Metabolic pathways	22 of 1433	4.04e-07
	csem04146	Peroxisome	7 of 91	2.04e-06
	csem00260	Glycine, serine and threonine metabolism	5 of 47	2.51e-05
Fosse nord down	csem03010	Ribosome	64 of 139	3.14e-56
	csem04141	Protein processing in endoplasmic reticulum	44 of 198	3.20e-27
	csem00970	Aminoacyl-tRNA biosynthesis	14 of 46	4.71e-10
	csem03013	RNA transport	20 of 170	6.26e-08
	csem04145	Phagosome	19 of 157	8.28e-08
	csem03050	Proteasome	11 of 50	6.81e-07
	csem00510	N-Glycan biosynthesis	11 of 58	2.20e-06
	csem03060	Protein export	7 of 21	1.56e-05
	csem01100	Metabolic pathways	57 of 1433	4.12e-05
Caging up	csem03040	Spliceosome	32 of 142	2.28e-22
	csem04145	Phagosome	17 of 157	2.15e-07
	csem04144	Endocytosis	24 of 325	2.15e-07
	csem04142	Lysosome	16 of 158	8.14e-07
	csem04141	Protein processing in endoplasmic reticulum	16 of 198	1.13e-05
	csem03008	Ribosome biogenesis in eukaryotes	10 of 72	1.61e-05
Caging down	csem01100	Metabolic pathways	89 of 1433	1.29e-42
	csem01200	Carbon metabolism	22 of 146	1.48e-16
	csem04146	Peroxisome	15 of 91	8.01e-12
	csem00980	Metabolism of xenobiotics by cytochrome P450	11 of 32	1.02e-11

csem00982	Drug metabolism - cytochrome P450	10 of 28	7.11e-11
csem00260	Glycine, serine and threonine metabolism	11 of 47	2.36e-10
csem01212	Fatty acid metabolism	10 of 58	2.01e-08
csem00983	Drug metabolism - other enzymes	10 of 57	2.01e-08
csem00500	Starch and sucrose metabolism	9 of 40	2.01e-08
csem00480	Glutathione metabolism	10 of 57	2.01e-08
csem00071	Fatty acid degradation	9 of 48	5.22e-08
csem00380	Tryptophan metabolism	8 of 46	5.71e-07
csem01230	Biosynthesis of amino acids	10 of 91	6.32e-07
csem00053	Ascorbate and aldarate metabolism	6 of 20	1.37e-06
csem03320	PPAR signaling pathway	9 of 78	1.63e-06
csem00670	One carbon pool by folate	6 of 21	1.63e-06
csem00630	Glyoxylate and dicarboxylate metabolism	7 of 37	1.63e-06
csem00010	Glycolysis / Gluconeogenesis	9 of 82	2.06e-06
csem00830	Retinol metabolism	7 of 46	5.39e-06
csem00040	Pentose and glucuronate interconversions	6 of 28	5.39e-06
csem00410	beta-Alanine metabolism	6 of 30	7.11e-06
csem00650	Butanoate metabolism	5 of 21	2.47e-05
csem00340	Histidine metabolism	5 of 22	2.88e-05
csem00100	Steroid biosynthesis	5 of 22	2.88e-05
csem00140	Steroid hormone biosynthesis	6 of 41	3.00e-05
csem00620	Pyruvate metabolism	6 of 44	4.16e-05
csem00280	Valine, leucine and isoleucine degradation	6 of 50	7.77e-05
csem00310	Lysine degradation	7 of 77	8.21e-05

*For these KEGG enrichment analyses, the protein sequences of the different datasets (up- and down-accumulated proteins in the livers of fish caged in Rouen and Fosse Nord (compared to fish caged in Canche), and fish caged in Canche (compared to fish stayed in natura)) were submitted to STRING v11.0 (Szklarczyk et al., 2019), and analyzed against the genome of the closest available pleuronectiform flatfish functionally annotated genome, ie. that of the tongue sole (*C. semilaevis*). For these analyses, we kept a FDR threshold of significance of 10^{-5}.

Table 3: List of the deregulated proteins potentially related to xenobiotics metabolism in caged fish: Rouen vs Canche, and Fosse Nord vs Canche.

accession	description	COG[1]	GO[2]	pI	mass	Mascot score	cove rage	Rouen vs Canche			Fosse Nord vs Canche		
								Tfold[3]	p value	PL[4] Class	Tfold[3]	p value	PL[4] Class
XP_019950625.1	NADPH--cytochrome P450 Reductase	C	-	5,3	77431	627	15,6	**1,85**	**1,29E-03**	**B**	-1,22	2,02E-01	R
XP_019935791.1	cytochrome P450 1A1	Q	+	6,1	59144	391	14,8	**4,26**	**9,53E-04**	**B**	1,43	1,57E-01	R
XP_019957767.1	cytochrome P450 2B4-like isoform X1	Q	-	7,1	55718	248	8,0	-1,21	1,18E-01	R	**-2,21**	**2,33E-04**	**B**
XP_019957775.1	cytochrome P450 2F2-like	Q	-	7,3	57472	282	7,8	1,38	1,64E-01	R	**-2,43**	**1,39E-02**	**B**
XP_019943606.1	cytochrome P450 2G1-like	Q	+	8,9	92626	540	13,1	**1,90**	**1,14E-04**	**B**	1,29	7,11E-02	R
XP_019943823.1	cytochrome P450 2G1-like	Q	+	8,1	56035	580	18,0	**2,48**	**1,29E-05**	**B**	1,30	1,88E-01	R
XP_019936551.1	cytochrome P450 2J2-like isoform X1	Q	-	7,7	56412	131	7,8	1,00	5,00E-01	R	**-2,00**	**4,07E-02**	**B**
XP_019936553.1	cytochrome P450 2J5-like	Q	-	5,9	56674	258	12,9	-1,46	3,21E-02	O	**-3,17**	**2,20E-04**	**B**
XP_019957326.1	cytochrome P450 2K1-like	Q	-	8,9	45368	138	8,5	1,37	4,01E-02	O	**-1,86**	**3,21E-02**	**B**
XP_019948310.1	cytochrome P450 4B1	Q	+	8,3	59094	120	7,8	**2,00**	**3,61E-02**	**B**	1,00	5,00E-01	R
XP_019962049.1	cytochrome P450 4V2	Q	-	7,3	58847	398	15,0	**2,16**	**3,63E-03**	**B**	**1,84**	**5,27E-03**	**B**
XP_019936740.1	dimethylaniline monooxygenase [N-oxide-forming] 5-like	Q	+	9,2	63433	420	11,9	**2,10**	**5,45E-05**	**B**	1,44	2,90E-02	O
XP_019947084.1	epoxide hydrolase 1	S	+	7,3	51845	163	11,2	**2,17**	**3,96E-03**	**B**	1,28	2,55E-01	R
XP_019965709.1	amine oxidase [flavin-containing]	Q	+	7,5	46946	499	19,7	**2,11**	**8,07E-04**	**B**	1,14	3,15E-01	R
XP_019940417.1	alcohol dehydrogenase 1-like	Q	+	7,0	40522	807	26,3	**1,53**	**2,84E-04**	**B**	1,17	7,44E-02	R
XP_019957877.1	aldehyde oxidase isoform X1	F	+	6,6	146085	1309	21,5	**1,86**	**3,84E-03**	**B**	1,16	2,80E-01	R
XP_019960923.1	quinone oxidoreductase	Q	+	6,5	34470	462	28,7	**-1,59**	**3,49E-04**	**B**	-1,06	2,64E-01	R
XP_019947438.1	glutathione S-transferase A4-like	O	+	7,6	25237	283	27,3	**1,69**	**2,94E-03**	**B**	1,26	7,34E-02	R
XP_019946255.1	glutathione S-transferase A-like	O	-	5,4	25664	157	17,0	**5,29**	**5,01E-04**	**B**	2,21	1,29E-01	G
XP_019950413.1	glutathione S-transferase omega-1	O*	-	6,3	27615	83	6,3	1,00	5,00E-01	R	**2,36**	**1,72E-02**	**B**
XP_019969171.1	UDP-glucuronosyltransferase 2A1-like, partial	C, G	+	6,1	42270	102	3,2	1,00	5,00E-01	R	**-1,75**	**2,34E-02**	**B**
XP_019954821.1	UDP-glucuronosyltransferase 2A2-like	G*	+	6,3	61267	176	8,1	**1,77**	**3,01E-04**	**B**	-1,30	9,39E-02	R

XP_019952519.1	UDP-glucuronosyltransferase 2C1-like isoform X1	G*	+	6,5	59864	261	7,5	**2,35**	**8,52E-04**	**B**	1,04	4,31E-01	R
XP_019969103.1	UDP-glucuronosyltransferase-like	G*	+	6,1	61449	100	6,2	**2,33**	**4,78E-03**	**B**	-1,25	3,00E-01	R
XP_019943023.1	mycophenolic acid acyl-glucuronide esterase, mitochondrial	S	-	6,9	32097	183	15,2	1,15	3,12E-01	R	**1,95**	**2,32E-03**	**B**
XP_019956774.1	bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthase 1	F	+	6,5	70243	148	5,6	-1,82	5,14E-02	G	**-2,00**	**4,61E-02**	**B**
XP_019952229.1	bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthase 2-like	F	+	6,6	69048	184	8,0	-1,09	3,44E-01	R	**-3,36**	**1,06E-04**	**B**
XP_019953221.1	3'(2'),5'-bisphosphate nucleotidase 1	F	+	5,7	33143	483	31,2	**-1,62**	**1,00E-02**	**B**	-1,21	1,39E-01	R
XP_019934383.1	3-oxo-5-beta-steroid 4-dehydrogenase-like	I*	-	5,9	32275	353	29,6	**2,28**	**2,81E-03**	**B**	1,44	1,89E-01	R
XP_019953837.1	adenylate kinase 4, mitochondrial	F	+	8,8	25462	133	10,6	1,06	4,35E-01	R	**3,00**	**2,49E-03**	**B**
XP_019966793.1	bile salt export pump-like	Q	-	7,2	145707	1734	23,8	-1,11	1,28E-01	R	**-2,52**	**4,93E-08**	**B**
XP_019944627.1	choline dehydrogenase, mitochondrial	C	-	8,7	70501	580	17,7	**2,35**	**2,61E-03**	**B**	**1,74**	**2,75E-02**	**B**
XP_019959810.1	hepatocyte nuclear factor 4-alpha isoform X1	K	+	6,7	50534	190	11,0	**-3,58**	**6,62E-06**	**B**	**-2,26**	**2,31E-04**	**B**
XP_019955942.1	long-chain-fatty-acid--CoA ligase 1-like isoform X1	I	+	6,2	80220	119	5,3	**2,10**	**3,04E-02**	**B**	1,00	5,00E-01	R
XP_019938496.1	L-xylulose reductase	Q	+	5,4	25675	168	13,9	**1,54**	**1,65E-02**	**B**	1,18	2,46E-01	R
XP_019938467.1	PHD finger-like domain-containing protein 5A	K	+	8,8	12397	201	33,6	**-2,62**	**1,04E-03**	**B**	-1,26	1,30E-01	R
XP_019956582.1	S-adenosylmethionine synthase isoform X1	H	+	5,8	43861	606	26,3	**-3,84**	**1,68E-04**	**B**	**-2,11**	**2,25E-03**	**B**
XP_019962483.1	thromboxane-A synthase-like, partial	Q	+	9,5	34268	112	9,7	**1,78**	**3,05E-02**	**B**	-1,15	3,11E-01	R
XP_019961561.1	uncharacterized protein LOC109641517 isoform X2	Q	+	6,0	27689	286	26,0	**-1,83**	**3,31E-04**	**B**	**-1,74**	**4,90E-03**	**B**
XP_019954768.1	betaine--homocysteine S-methyltransferase 1	E	-	6,1	43735	1248	50,4	-1,10	1,37E-01	R	**-1,52**	**1,22E-04**	**B**
XP_019954766.1	betaine--homocysteine S-methyltransferase 1-like	E	-	7,2	44205	1608	61,1	1,19	1,10E-01	R	**-1,74**	**1,89E-03**	**B**
XP_019956435.1	betaine--homocysteine S-methyltransferase 1-like	E	-	6,0	41923	508	27,2	5,00	1,78E-05	B	**2,52**	**1,75E-03**	**B**

XP_019935095.1	adenosylhomocysteinase 2-like isoform X1	H	-	5,8	63240	157	3,3	1,00	5,00E-01	R	**4,20**	**4,27E-03**	**B**
XP_019958954.1	cystathionine beta-synthase-like	E	-	7,9	64547	882	31,1	1,01	4,68E-01	R	**1,84**	**6,23E-06**	**B**

[1]: COG, Cluster of Orthologous Groups; all assignations were manually checked; asterisked categories were manually assigned.

[2]: GO, Gene Ontology. + and – refer to proteins that were assigned at least one of the GO terms related to xenobiotics metabolism, i.e. GO:0006805 (xenobiotic metabolic process) or GO:0042178 (xenobiotic catabolic process) or GO:0042908 (xenobiotic transport) or GO:0052697 (xenobiotic glucuronidation) or GO:0009410 (response to xenobiotic stimulus) or GO:0071466 (cellular response to xenobiotic stimulus)

[3]: TFold : + and – to refer to proteins up- or down-accumulated in the livers of fish encaged in Rouen or Fosse Nord vs in the Canche estuary.

[4]: PatternLab's classes as given by the TFold module; Only the Blue class (B, bolded) identifications met both the Tfold (≥1.5) and statistical criteria (p value ≤0.05). Orange (O), green (G) and red (R) ones were thus disconsidered.

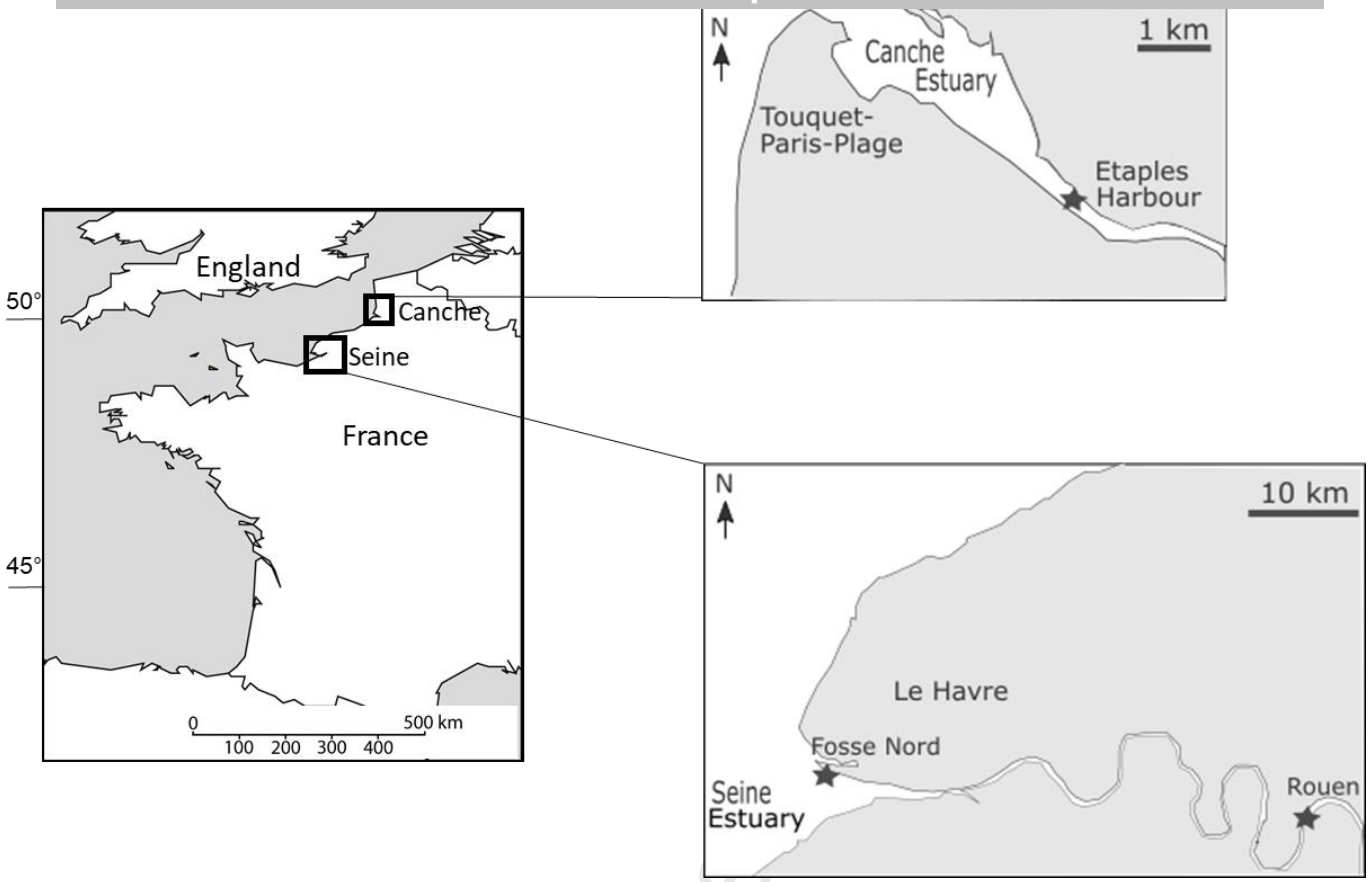


Fig. 1 : Sampling and caging sites of juvenile flounders in Canche and Seine estuaries.

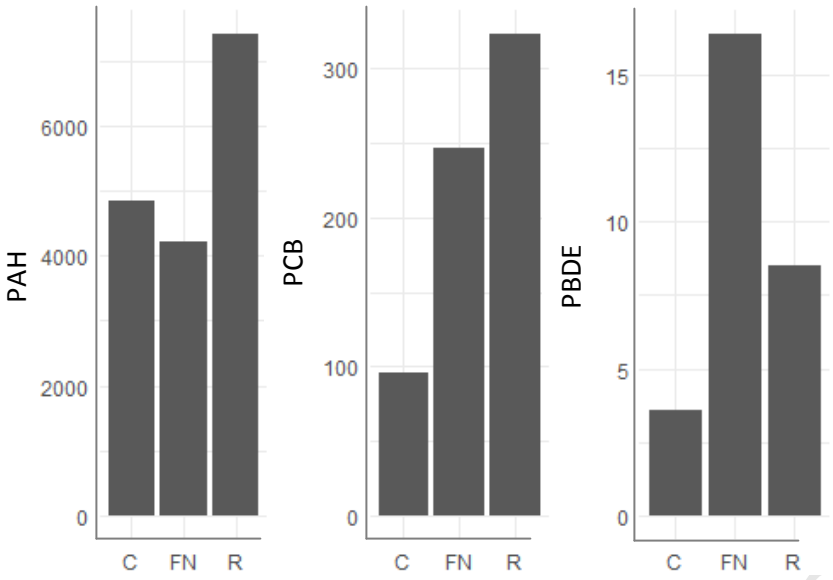


Fig. 2: PAHs, PCBs and PBDEs (in $ng.g^{-1}$) concentrations in sediment of Canche (C), Fosse Nord (FN) and Rouen (R).

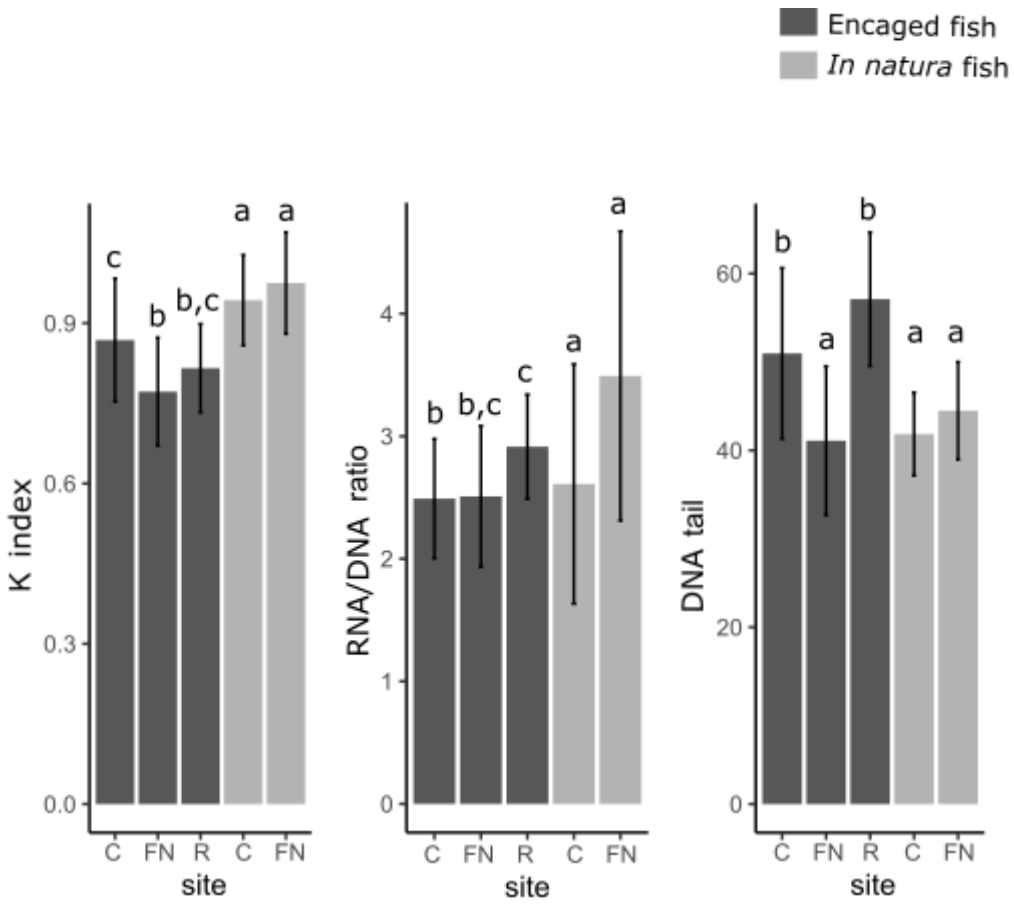


Fig. 3: Phenotypic indicators results in Canche (C), Fosse Nord (FN), Roue n (R) for caged fish (dark grey) and fish caught in natura (light grey): physiological status of fish was estimated by measuring the Fulton condition index (K index), metabolic activities measured on muscle (RNA/DNA ratio), primary DNA damage levels expressed as the mean tail moment measured by the Comet assay on erythrocytes (DNA tail).

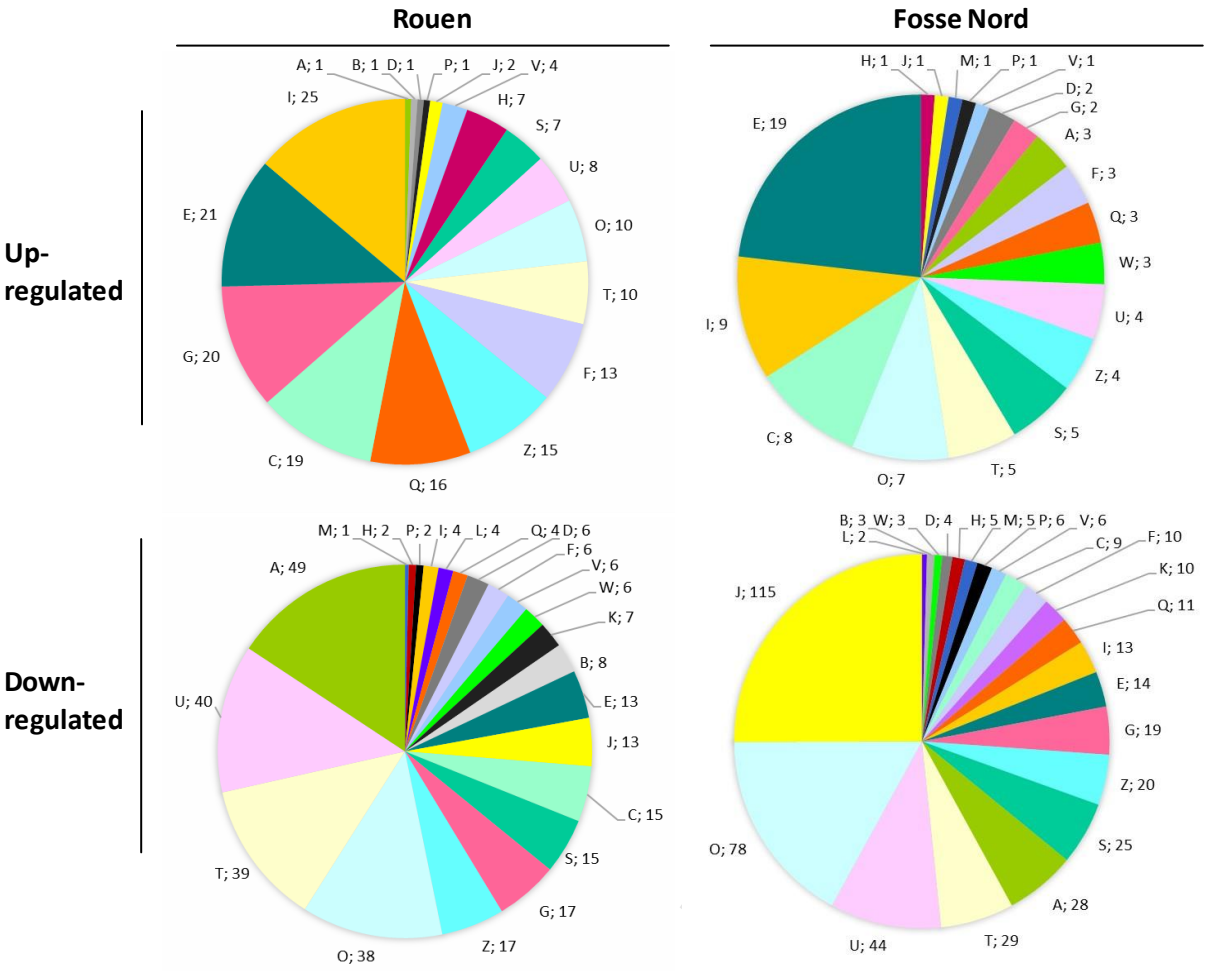


Fig. 4 : Pie charts representing the main Clusters of Orthologous Group (COG) categories that are up-regulated and down-regulated in Rouen- (left) and Fosse Nord- (right) caged fish compared to fish caged in the Canche estuary : metabolism of lipids (I), amino acids (E), carbohydrates (G), energy production and conversion (C), RNA processing and modification (A), intracellular trafficking (U), signal transduction mechanisms (T), post-translational modifications, protein turnover and chaperones (O), and translation, ribosomal structure and biogenesis (J)

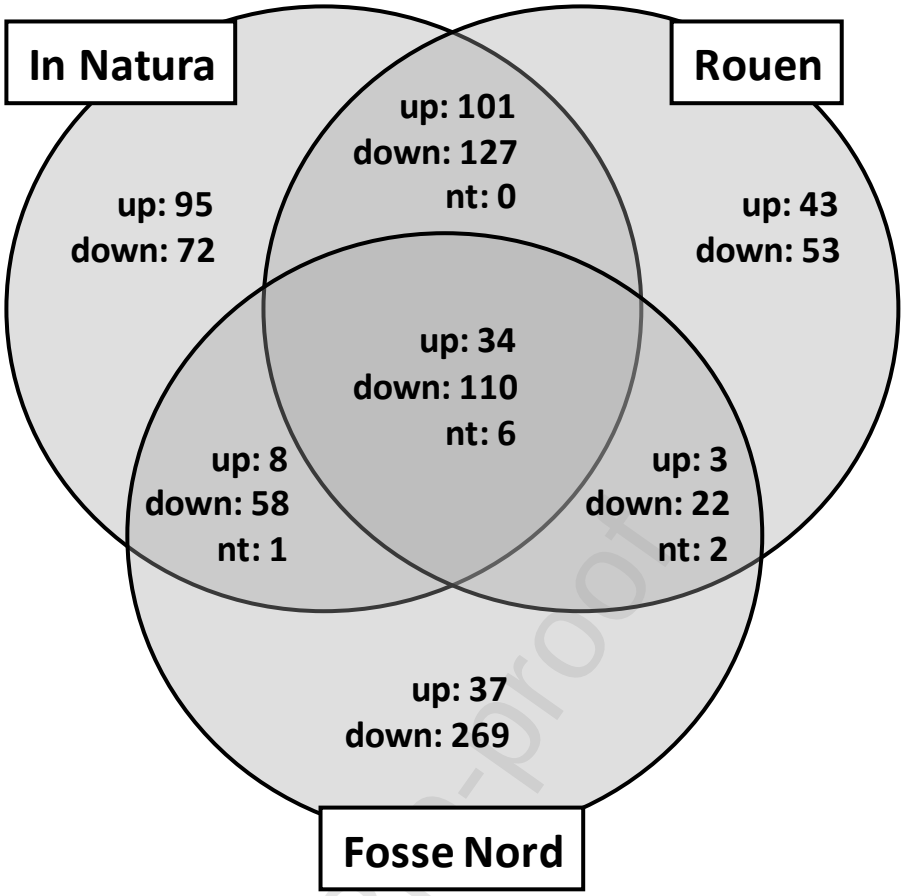
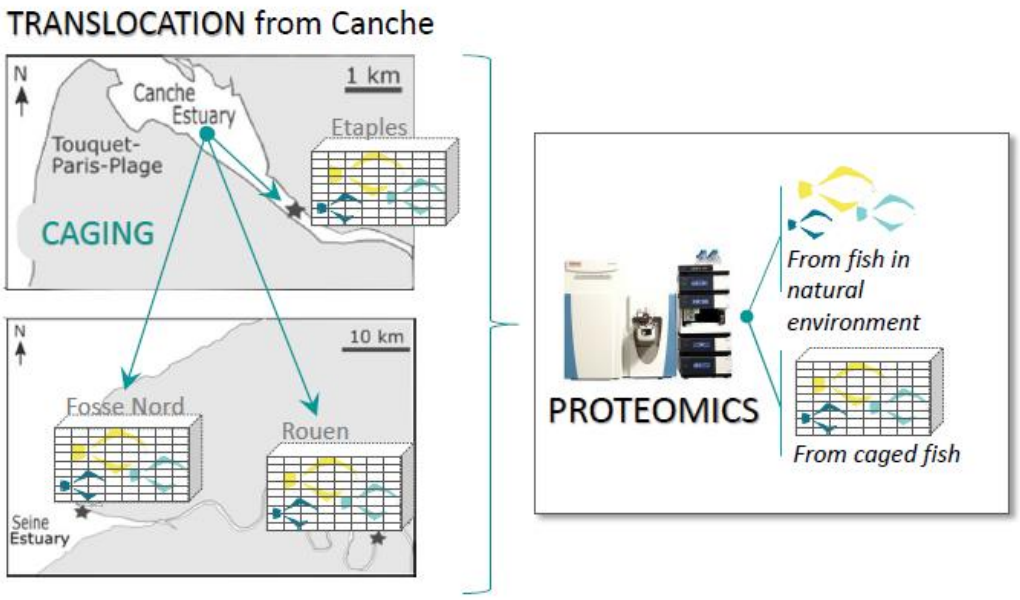


Figure 5: Comparison of the three subsets of proteins found deregulated in the livers of fish encaged in Rouen and Fosse Nord, and caught *in natura*, as compared to fish encaged in the Canche estuary. Numbers correspond to proteins showing the same accumulation trend (up or down). nt: no trend (one up, and one down).



Graphical abstract

HIGHLIGHTS:

- Platichthys flesus juveniles were caged in two differentially polluted sites of the Seine estuary
- the liver proteome of fish phenotypes were determined
- proteins implied in xenobiotics detoxification were positively deregulated in the most polluted area
- control fish (caged in Canche) overexpressed many stress proteins
- caging is an interesting technique to evaluate estuarine water quality at a micro-scale