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

3
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13
14
15 **Abstract**

16
17 Estuaries are important areas highly vulnerable to anthropogenic pollutions. Therefore, the
18 assessment of estuarine water quality is a major ecological issue. In this study, we sampled
19 juveniles of the European flounder in the “pristine” Canche estuary, and caged them in Canche
20 and in two polluted sites of the Seine estuary, Rouen and Fosse Nord. After one month, the
21 metal and organic pollutants in these sites were assessed, and we evaluated several phenotypic
22 indicators (condition index, RNA/DNA ratios and genotoxicity), and extracted the proteins in
23 fish livers for analysis using a shotgun proteomics approach.

24 The results showed strong modifications in the fish caged in both sites of the Seine estuary, as
25 compared to those caged in Canche. In particular, many proteins involved in phase I and phase

26 II detoxification reactions were accumulated in the liver of fish caged in the site showing the
27 highest pollution, Rouen. In addition, we observed a general disruption of metabolism, in
28 particular an increase in lipid synthesis and carbohydrate degradation in Rouen, and a decrease
29 in the abundance of proteins associated to translational activity in Fosse Nord. At both sites,
30 several stress proteins were decreased.

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

38

39

40 **Introduction**

41 Estuaries are among the most productive ecosystems over the world, giving them a
42 high ecosystemic value (Costanza *et al.*, 1997; Courrat *et al.*, 2009). These coastal ecosystems
43 are generally considered as major nurseries for many marine species (Chapman & Wang,
44 2001). Nowadays, estuaries are heavily impacted by anthropogenic activities, such as resource
45 overexploitation, pollution, or habitat fragmentation (Aubry & Elliott, 2006; Lotze *et al.*, 2006).
46 Monitoring the overall quality of estuarine habitats, especially those acting as fish nurseries, is
47 therefore of utmost importance to preserve marine resources (Beck *et al.*, 2001).

48 The Seine estuary is one of the most anthropogenically polluted estuaries in Europe
49 (Carpentier *et al.*, 2002; Cachot *et al.*, 2006; Burgeot *et al.*, 2017). Its basin covers more than a
50 quarter of the French population and includes major harbours, industrial and agricultural
51 areas (Mouny & Dauvin, 2002; Dévier *et al.*, 2013; Erraud *et al.*, 2018). Thus, the Seine estuary
52 is subjected to eutrophication, organic and metallic contaminations (Blanchet *et al.*, 2008;
53 Dauvin, 2018). Furthermore, this estuary is particularly concerned by degradation and loss of
54 flatfish nursery habitats, thus displaying a reduced capacity for the recruitment and growth of
55 juvenile fish (Riou *et al.*, 2001, Le Pape *et al.* 2007; Rochette *et al.*, 2010; Archambault *et al.*,
56 2015; Tecchio *et al.*, 2016).

57 The quality of nursery can be measured by density, survival and growth of juveniles,
58 and by the possibility for fish to move towards subadult habitats (Beck *et al.*, 2001; Schloesser &
59 Fabrizio, 2018). The growth and survival of fish depend directly of prey availability and
60 predation, but also of hydrological conditions and anthropogenic factors (Amara *et al.*, 2007,
61 2009). A large panel of pollutants (metals, PAHs, PCBs, ...) are monitored in the Seine estuary,
62 and numerous studies explored the resident wild life responses to xenobiotics in this highly
63 polluted ecosystem (Ohe *et al.*, 2004; Marchand *et al.*, 2004; Galland *et al.*, 2013; Dupuy

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

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

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

85

86

87 **Materials & Methods**

88 ***Study sites***

89 The study area was located along the French coast of the English Channel (Fig. 1). Two
90 estuaries were investigated (Canche & Seine). The Canche is a small estuary with low freshwater
91 input of $7 \text{ m}^3 \cdot \text{s}^{-1}$; it is located near a protected area and is not impacted by important human
92 activities and is considered as a “pristine estuary” (Amara *et al.*, 2009). The Seine estuary is the
93 largest estuary along the English Channel, concentrating the quarter of the French population,
94 one third of the French industry and intensive agriculture (Mouny & Dauvin, 2002). The
95 freshwater input varies from around $100\text{-}200 \text{ m}^3 \cdot \text{s}^{-1}$ in summer, to the maximum recorded in
96 winter: $2,000 \text{ m}^3 \cdot \text{s}^{-1}$ (Mouny *et al.*, 1998). In spite of significant efforts to restore the
97 environmental quality of the Seine estuary during the past few decades, it remains one of the
98 most chemically polluted estuaries in western northern Europe (Dauvin *et al.* 2007; Poisson,
99 2011).

100 ***Sampling and caging experiment***

101 In September 2017, 150 0-group juvenile flounders *Platichthys flesus* (7–9 cm total
102 length: TL) were collected in the Canche estuary by a small beam trawl; 30 juvenile flounders
103 being also collected in the Seine estuary according to the same procedure (Fosse Nord site). A
104 set of 30 fish per estuary were immediately analyzed after their catch in Canche and Seine; the
105 objective was to compare the responses of flounders sampled in natura vs caged flounders.
106 The remaining fish (*i.e.* 120 individuals from the Canche) were acclimatized for 1 week in a
107 500-L aquarium supplied with an open seawater circuit and were fed daily with frozen
108 Mysidacea and brine shrimps (*Artemia* sp.). One day before the caging experiment,
109 flounders were anesthetized (eugenol 35 mg/L), weighed (to the nearest 1 mg), measured
110 for total length (within 0.1 mm), and individually marked (visual implant tag, $1.2 \text{ mm} \times 2.7$

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

114 Cage settlement was carried out on the 12th and 13th of September 2017 at three
115 different sites. Two sites were chosen in the Seine estuary: Rouen (49° 22.995' N; 01° 00.676'
116 E), which is located near a wastewater treatment plant (Emeraude), and Fosse Nord (49°
117 27.328' N; 00° 07.493' E), which is located in the main channel of the estuary (Fig. 1). The last
118 caging site was located in the Canche estuary, close to the Etaples harbour (50° 30.982' N; 01°
119 37.852' E) (Fig. 1). Three cages were settled by site, each containing between 15 and 20 fish.

120 The cages were made of plastic. Their length was 1 m, whereas their width and height
121 were 0.6 m. Their 15 mm mesh size facilitated water circulation. The cages were fixed to the
122 bottom with two screw anchors secured by scuba divers, the average depth being 8 m and 3 m,
123 respectively, in the Seine and Canche estuaries; these depths are representative of the highest
124 densities of juvenile flounders in the two estuarine systems (Amara *et al.*, 2009). After the one
125 month caging, all fish were quickly transferred to the laboratory and then killed by cervical
126 dislocation, the method classically used on juvenile flatfish (Dupuy *et al.*, 2015); they were
127 identified by their tags, weighed and measured. In order to evaluate the potential effect of
128 contaminants on juvenile fish, we calculated the Fulton's condition index (K), considered as an
129 indicator of the fish general well-being (Minier *et al.*, 2015). K was assessed with the formula :
130 $k = 100 * (W/L^3)$ where W= gutted body weight (i.e. carcass without internal organs) (g) and L =
131 total length (cm) (Peig & Green, 2010). The same protocol was conducted on flounders trawled
132 *in natura* (Canche and Seine), immediately after the catch. The liver and a blood sample were
133 collected per fish, then flash-frozen in liquid nitrogen.

134

135 ***Pollutants analysis***

136 *Sample preparation and metals analysis in sediment*

137 Dry sediment (200 mg) was totally digested in a heating block (HotBlock® SC100 Digestion
138 System), with an acid mixture of aqua regia (HNO₃/HCl 1:3, v/v) and HF (10 mL) following a
139 procedure described by Ouddane et al. (1999) with some modification. The sample was
140 weighed into PTFE digestion cups, when the sediments were totally dissolved at 120 °C, the
141 solution was then diluted with ultrapure Milli-Q+ water (18.2 MΩ cm) and filtered through a
142 0.45 µm cellulose acetate membrane. The metal concentrations were determined by
143 inductively coupled plasma-atomic emission spectrometry ICP-AES (Agilent 5110, dual view) for
144 the major element and by ICP-MS (Varian, 820) for the trace elements. Procedural blanks and
145 certified reference materials (CRMs) MESS-3, HISS-1 and PACS-2 (National Research Council of
146 Canada) were prepared using the same analytical procedure and reagents and analysed for
147 quality assurance/quality control. All results were >92% confidence intervals.

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

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

161 *Organic pollutants in sediment*

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

180 **Genotoxicity**

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

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

190 ***Shotgun proteomics***

191 *Proteomic samples preparation*

192 We chose to analyse the liver proteomes of fish encaged in the different estuaries
193 because the liver is the main organ where detoxification metabolism occurs. Frozen livers
194 obtained from 5 fish collected *in natura* (Canche) and 5 caged fish per site (Canche, Rouen,
195 Fosse Nord) were each homogenized in 1 ml of Tris-HCl buffer (100 mM, pH 6.8) containing 1%
196 Protease Inhibitor Mix (GE Healthcare), using a Precellys 24 system (Bertin Technologies). The
197 supernatants were removed by centrifugation (12 000 g, 15 min, 4°C) and then incubated at
198 ambient temperature for 30 min with 1% DNase mix (GE Healthcare). Total protein contents
199 were determined using DC protein assay kit (biorad) in triplicate. A quantity of 30 μ g of proteins
200 was treated as previously described (Hartmann et al., 2014). Briefly, proteins were subjected to
201 a short SDS-PAGE migration on a 4–12 % gradient 10-well NuPAGE gel (Invitrogen). The
202 polyacrylamide bands corresponding to each whole proteome were excised. Proteins were first
203 reduced with DTT and alkylated with iodoacetamide, and then proteolyzed with Trypsin Gold
204 (Promega) in presence of 0.01 % ProteaseMAX surfactant (Promega) in 50mM NH₄HCO₃ for 1 h
205 at 50 °C. Then, a volume of TFA 5 % was added to reach a final concentration of 0.5 %.

206 *Q Exactive HF analysis*

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

219 *Proteomic data interpretation*

220 The recorded MS/MS spectra were searched with MASCOT against the *Paralichthys*
221 *olivaceus* full annotated proteome database (Shao et al., 2017) which comprises 36,123
222 polypeptide sequences totaling 22,711,544 residues (GCF_001970005.1 v1.0, downloaded
223 2018-01-19). The following parameters were used: tryptic peptides with a maximum of 2
224 miscleavages during proteolytic digestion, a mass tolerance of 5 ppm on the parent ion and
225 0.02 Da on the MS/MS, fixed modification for carbamidomethylated Cys (+57.0215) and
226 variable modification for oxidized Met (+15.9949) and deamidated Asn or Gln (+0.9840). All
227 peptide matches with a peptide score above its query identity threshold set at $p < 0.05$ with
228 the database and rank 1 were parsed using the IRMa 1.31.1c software (Dupierris et al., 2009). A
229 protein was considered validated when at least two different peptides were detected. False-
230 positive identification of proteins were estimated using a reverse decoy database as below

231 0.1% with these parameters.

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

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

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

259 *Proteomic data deposition*

260 The mass spectrometry proteomics data have been deposited to the ProteomeXchange
261 Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository
262 (Perez-Riverol et al., 2019) with the dataset identifier PXD013684 (project DOI:
263 10.6019/PXD013684).

264

265 **Results and discussion**

266 ***Pollutants analysis***

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

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

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

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

295

296 ***Phenotypic indicators***

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

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

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

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

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

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

343

344 ***Shotgun proteomic approach***

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

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

356 In total, almost 3 million MS/MS spectra (2,980,656) were recorded, with an average of
357 74,516 MS/MS spectra per run. We analysed these spectra against the protein database of
358 *Paralichthys olivaceus*, a flatfish species closely related to *P. flesus* with a fully sequenced
359 genome (Shao *et al.*, 2015), and we could assign a total of 853,691 spectra to peptide
360 sequences (Supplementary data, Table S1). On average, about 21,000 MS/MS spectra per
361 run could be interpreted, i.e. an attribution rate of 28%, which is quite usual for a non-
362 model organism and the use of a heterologous protein database. In total, no less than
363 3,761 proteins could be identified, including 2,511 certified by at least 2 different peptides
364 (Table S2), on which the comparative statistical analysis was carried out using the PatternLab
365 software. The analyses were carried out in pairs, each time using the fish caged in the Canche
366 estuary as a reference, and applying as the thresholds of significance a p value of 0.05 and
367 an amplification (or reduction) factor of 1.5 for protein abundance.

368

369 ***Proteomic signatures of the fish encaged in Rouen and Fosse Nord***

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

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

380

381 *Functional analysis of deregulated proteins*

382 Functional analysis makes it possible to visualize the main modifications and
383 orientations of the metabolism under the different experimental conditions. To this end, the
384 various protein subgroups were re-annotated using Blast2GO and EggNOG (Conesa *et al.*,
385 2005; Powell *et al.*, 2014) softwares, followed by a careful manual re-examination. Results
386 presented in Figure 4 show the number of proteins assigned to the different Clusters of
387 Orthologous Group (COG) categories. They show that the main categories accumulated in the
388 liver from fish caged in Rouen were related to the transport and metabolism of lipids (I), amino
389 acids (E) and carbohydrates (G), as well as energy production and conversion (C). Concerning
390 the Fosse Nord site, a single category clearly dominated the subgroups of accumulated
391 proteins, *i.e.* the transport and metabolism of amino acids (E). It was followed by the categories I
392 (lipid metabolism and transport) and C (energy production and conversion). For down-
393 accumulated proteins (Fig. 4), the result were also very clear in fish caged in Fosse Nord,
394 with three clearly dominant categories, *i.e.* translation, ribosomal structure and biogenesis
395 (J), suggesting an overall depression of translational activity in cells, followed by categories O
396 (PTM: post-translational modifications, protein turnover and chaperones) and U (intracellular
397 trafficking), both related to protein processes and functioning. In the fish encaged in Rouen,
398 the latter two categories were also affected, but the overall changes appeared more

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

402 In order to go deeper in this functional analysis, we carried out KEGG enrichment analyses of all
403 the deregulated protein subsets identified in this study, using the pleuronectiform flatfish genome
404 *Cynoglossus semilaevis* as a reference. The results presented in Table 2 show a very significant
405 enrichment of the pathways involved in the detoxification of drugs and xenobiotics (KEGG pathways
406 00982, 00980 and 00983) in the group of proteins accumulated by fish caged in Rouen. It is noteworthy
407 that these pathways, which do not appear enriched in the livers of fish caged in Fosse Nord, seemed
408 rather reduced in our study on the impact of caging, which only concerned fish caged (or not) in our
409 control estuary (Canche). This analysis also suggested the stimulation of the peroxisomal lipids
410 metabolism (csem04146) in the same condition (Rouen), as well as in fish caged in Fosse Nord, as
411 compared to those encaged in Canche. For the rest, the overall results are very consistent with the
412 previous COG analysis. These results are particularly interesting. Bearing in mind that the two
413 polluted sites are in the same Seine estuary, but that the Rouen site has more metal and organic
414 contamination (PCBs and PAHs), while the Fosse Nord site mainly contains PBDEs, we can
415 expect common effects and different effects on fish caged at the two sites. Going deeper into
416 the mechanisms, we can see more precisely how fish manage these different situations.

417

418 *Deregulation of xenobiotic metabolism*

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

424 The Phase I mechanisms usually involve chemical reactions of hydrolysis, reduction and
425 oxidation, exposing or introducing a functional group (-OH, -NH₂, -SH or -COOH) increasing the
426 reactivity and hydrophilicity of the molecule. One major protein system involved in these
427 reactions is represented by Mixed Function Oxidases (MFO), which require the coordinated
428 action of a cytochrome P450 protein, together with a NADPH-Cytochrome P450 Reductase
429 (Livingstone, 1998). These systems are often used to assess the ecotoxicological status of
430 ecosystems, by measuring ethoxyresorufin-O-deethylase (EROD) activity, which is a recognized
431 biomarker of the presence of pollutants such as PAHs, PCBs and dioxins for example (Porte *et*
432 *al.*, 2000; van der Oost *et al.*, 2003).

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

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

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

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

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

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

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

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

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

520

521 *General perturbation of the cell metabolism*

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

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

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

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

547 Finally, the most significant point at Fosse Nord is linked to translation, as more than
548 100 proteins related to translation, ribosomal structure and biogenesis were down-
549 accumulated, thus suggesting a general inhibition of the cell translational system. Similar
550 response has already been described in response to a variety of stressing situations (Brostrom
551 *et al.*, 1996; von Born *et al.*, 2018). In these situations, mRNAs are generally stored in specific
552 structures, the so-called "stress granules", which include both poly(A)+ mRNA, ribosomal
553 proteins (mainly 40S ribosomal subunits), eIF elongation factors, and PABP (polyadenylate
554 binding protein, many elongation factors) (Mazroui *et al.*, 2006; Anderson & Kedersha 2006).
555 Most of these proteins were decreased in our study.

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

568 same environment.

569

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

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

584 All fish came from the same population, and shared the same natural ecosystem, i.e.
585 the Canche estuary, both before and during the caging period. One could therefore have
586 expected minimal differences between the two conditions (encaged or not, in the same
587 estuary). In fact, the results showed that some 605 proteins were significantly deregulated
588 between the two conditions, which corresponded to 16% of the total observed proteome
589 (3,774 proteins identified). Of these proteins, 367 were accumulated, and 238 showed a
590 reduced abundance, in the livers of encaged fish as compared to the *in natura* condition (Table
591 S2). Fish physiology seems therefore to be deeply modified in this situation of encagement.

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

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

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

616 the post-transcriptional level, under caging conditions.

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

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

638

639 ***Concluding remarks***

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

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

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

675

676

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

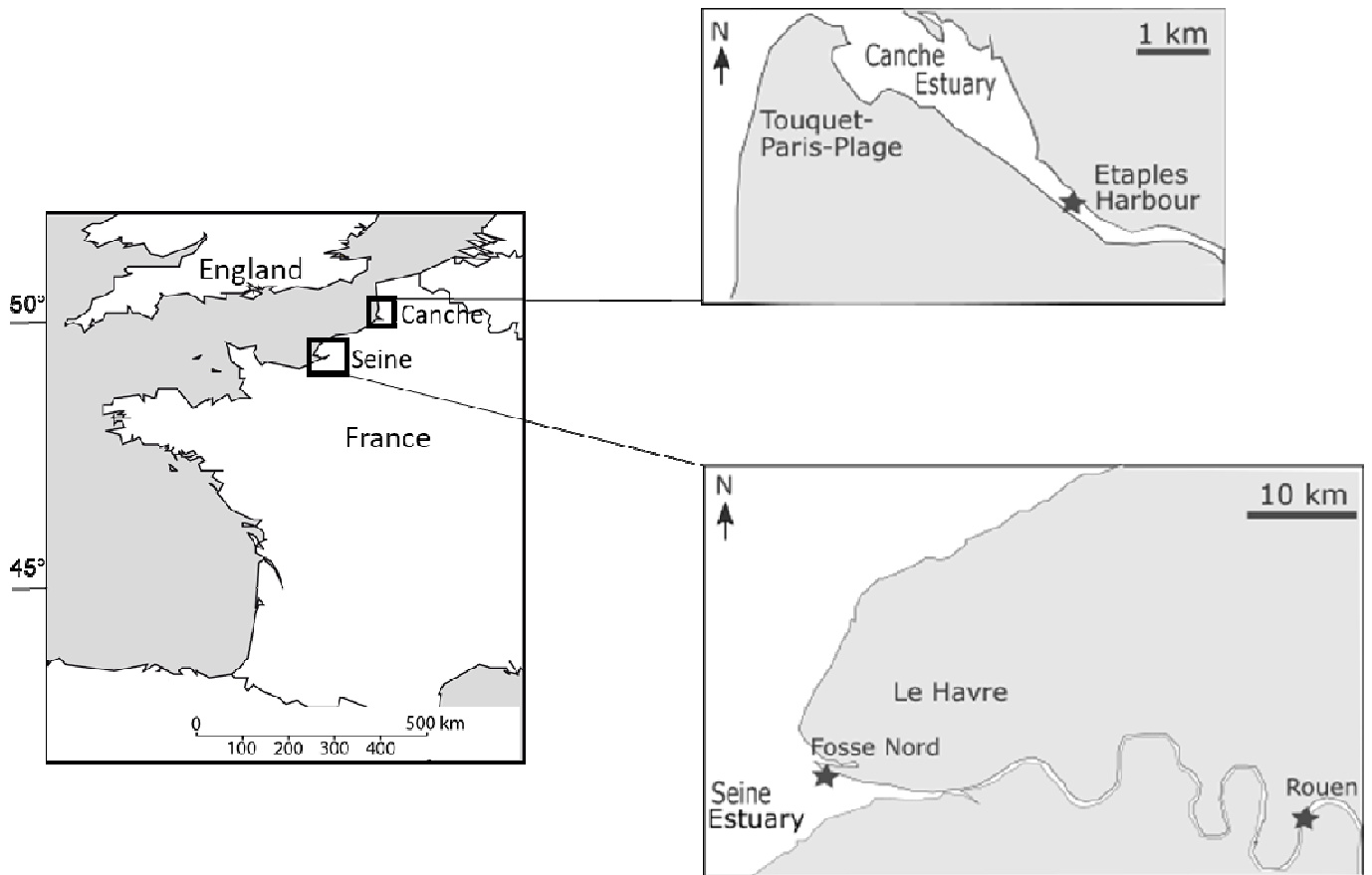


Fig. 1 : Sampling and caging sites of juvenile flounders in Canche and Seine estuaries.

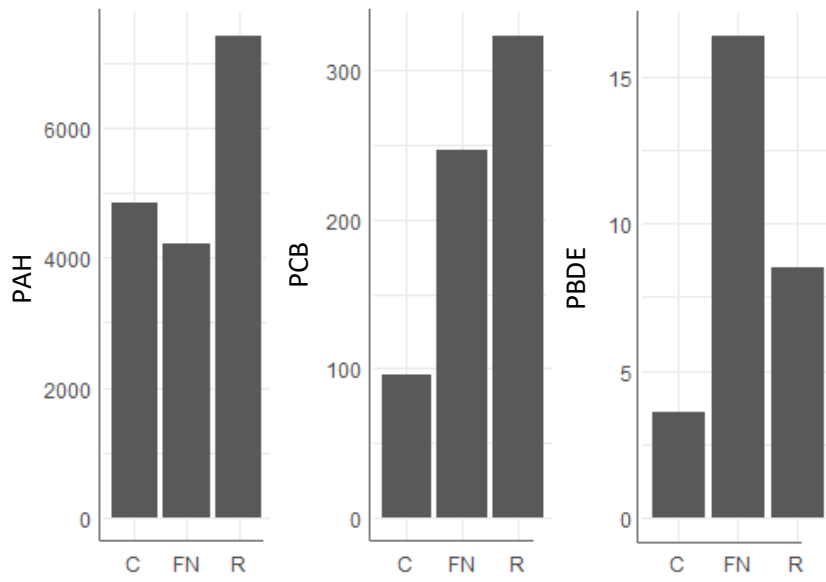


Fig. 2: PAHs, PCBs and PBDEs (in ng.g^{-1}) concentrations in sediment of Canche (C), Fosse Nord (FN) and Rouen (R).

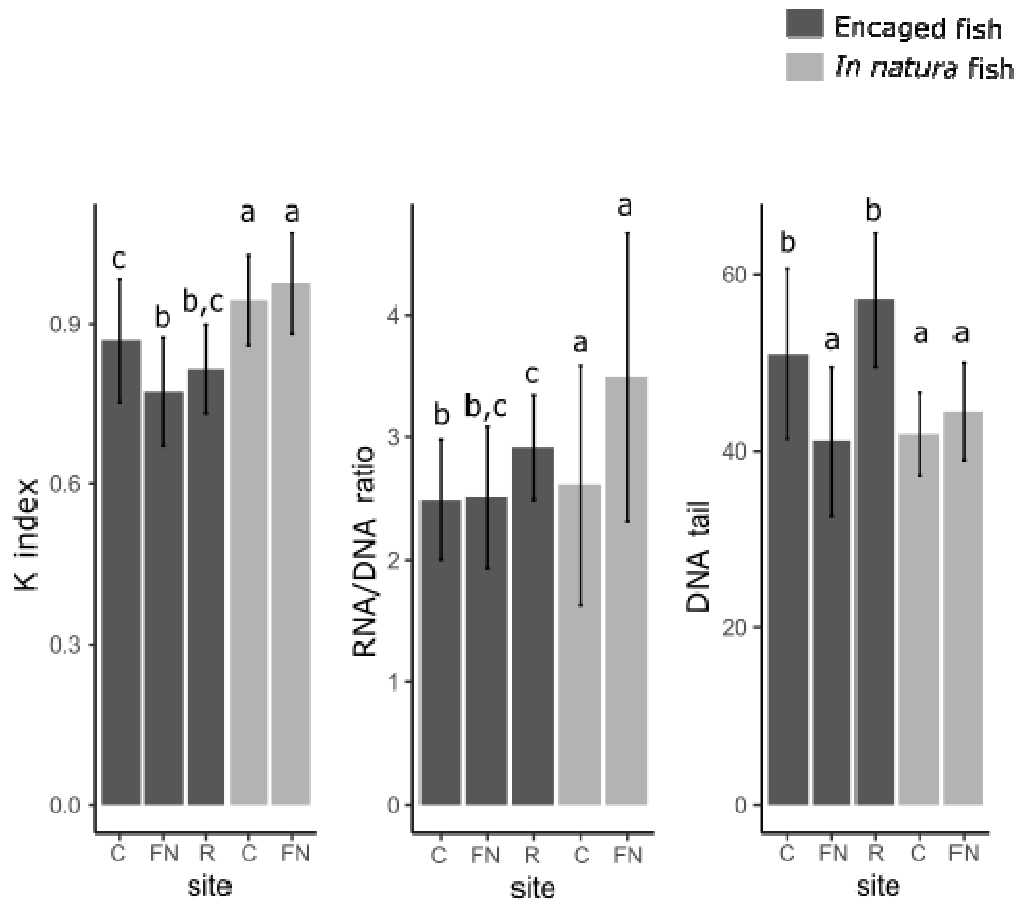


Fig. 3: Phenotypic indicators results in Canche (C), Fosse Nord (FN), Rouen (R) for caged fish (dark grey) and fish caught in natura (light grey): physiological status of fish was estimated by measuring the Fulton condition index (K index), metabolic activities measured on muscle (RNA/DNA ratio), primary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

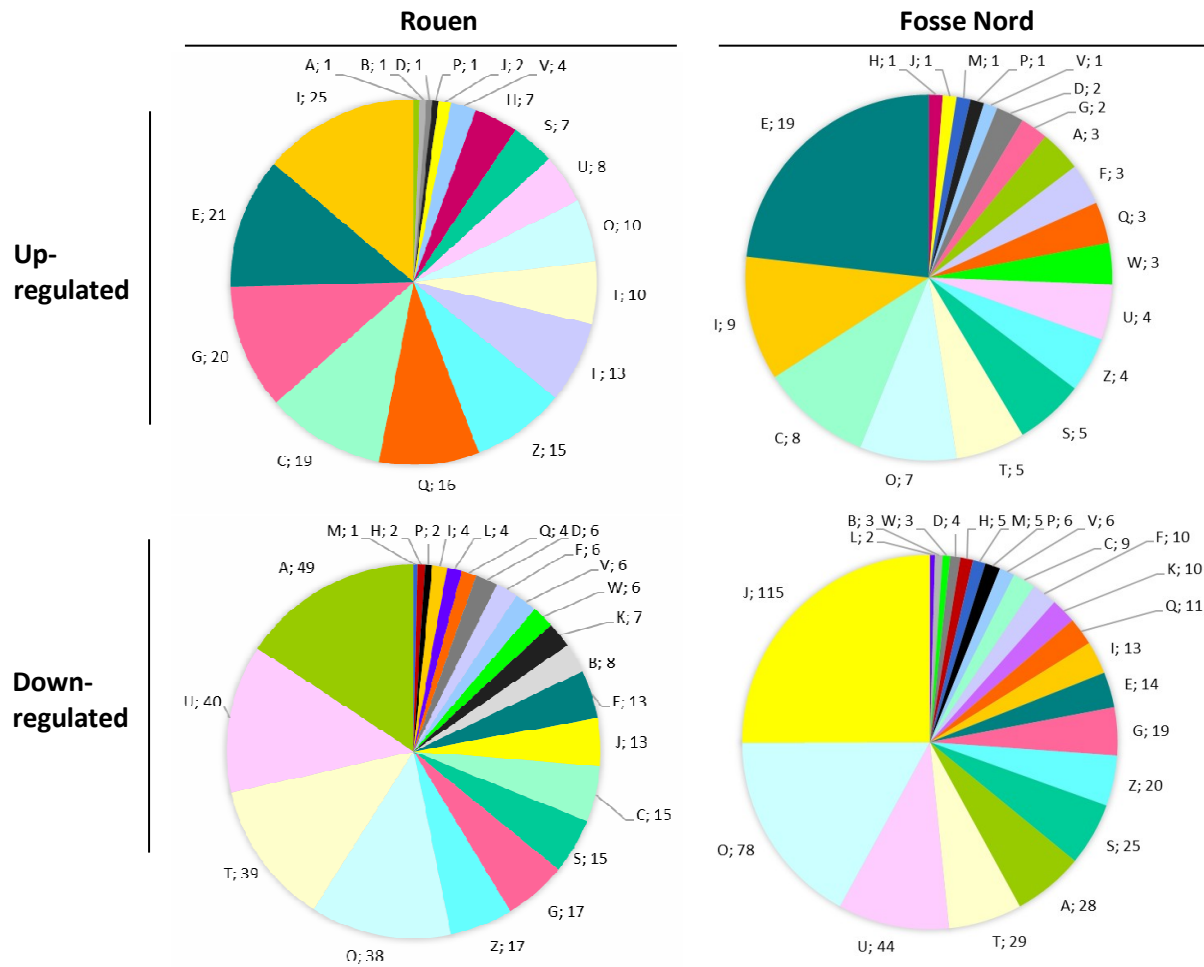


Fig. 4 : Pie charts representing the main Clusters of Orthologous Group (COG) categories that are up-regulated and down-regulated in Rouen- (left) and Fosse Nord- (right) caged fish compared to fish caged in the Canche estuary : metabolism of lipids (I), amino acids (E), carbohydrates (G), energy production 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)

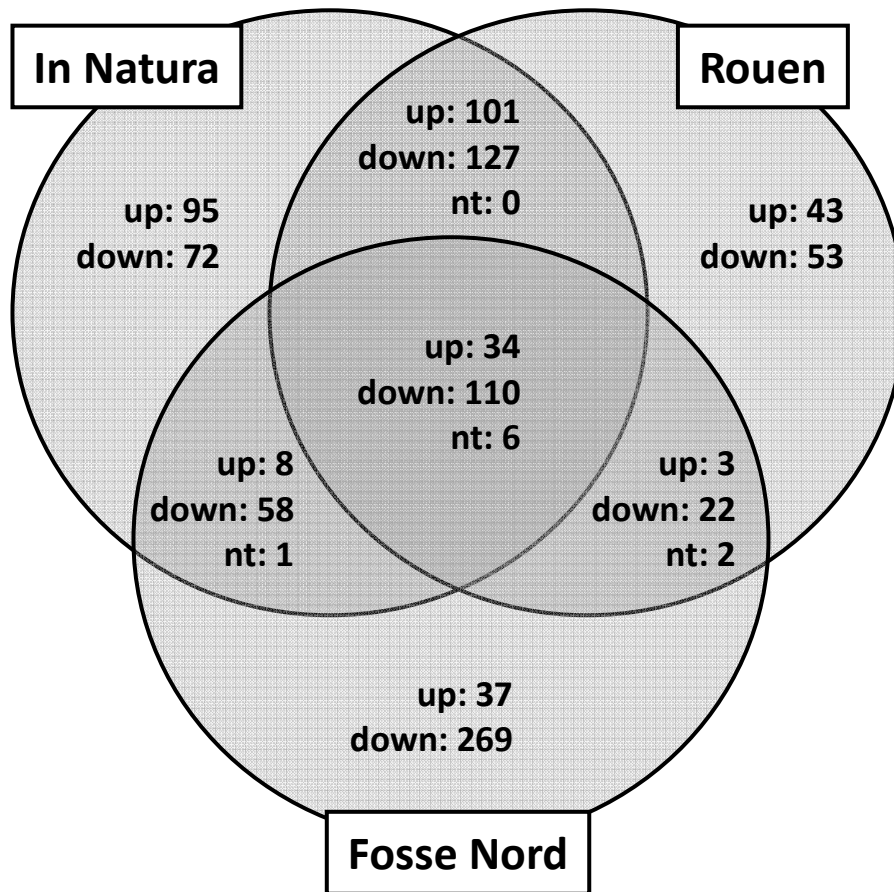


Figure 5: Comparison of the three subsets of proteins found deregulated in the livers of fish encaged in Rouen and Fosse Nord, and caught *in natura*, as compared to fish encaged in the Canche estuary. Numbers correspond to proteins showing the same accumulation trend (up or down). nt: no trend (one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

TRANSLOCATION from Canche

