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# Environmental signatures and fish proteomics: a multidisciplinary study to identify the major stressors in estuaries located in French agricultural watersheds

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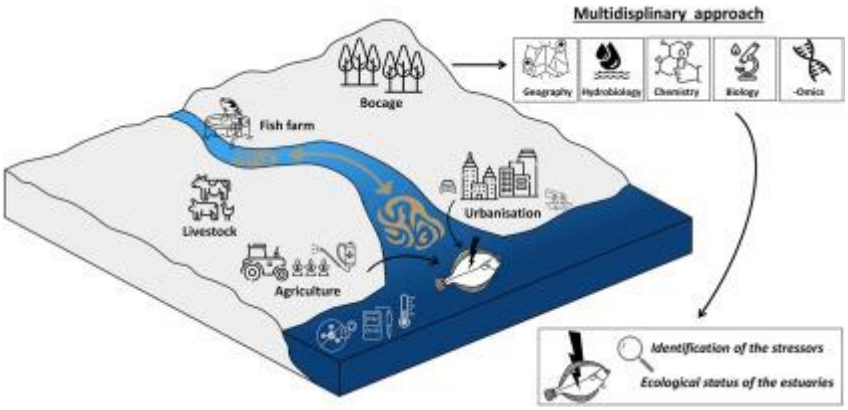
**Abstract :**

Watersheds and estuaries are impacted by multiple anthropogenic stressors that affect their biodiversity and functioning. Assessing their ecological quality has consequently remained challenging for scientists and stakeholders. In this paper, we propose a multidisciplinary approach to identify the stressors in seven small French estuaries located in agricultural watersheds. We collected data from landscape (geography, hydrobiology) to estuary (pollutant chemistry) and fish individual scales (environmental signatures, proteomics). This integrative approach focused on the whole hydrosystems, from river basins to estuaries. To characterize each watershed, we attempted to determine the land use considering geographic indicators (agricultural and urbanised surfaces) and landscape patterns (hedges density and riparian vegetation). Juveniles of European flounder (Platichthys flesus) were captured in September, after an average residence of five summer months in the estuary. Analyses of water, sediments and biota allowed to determine the concentrations of dissolved inorganic nitrogen species, pesticides and trace elements in the systems. Environmental signatures were also measured in flounder tissues. These environmental parameters were used to establish a typology of the watersheds. Furthermore, data from proteomics on fish liver were combined with environmental signatures to determine the responses of fish to stressors in their environments. Differential protein abundances highlighted a dysregulation related to the detoxification of xenobiotics (mainly pesticides) in agricultural watersheds, characterized by intensive cereal and vegetable crops and high livestock. Omics also revealed a dysregulation of proteins associated with the response to hypoxia and heat stress in some estuaries. Furthermore, we highlighted a dysregulation of proteins involved in urea cycle, immunity and metabolism of fatty acids in several systems. Finally, the combination of environmental and molecular signatures appears to be a relevant method to identify the major stressors operating within hydrosystems.

## Graphical abstract




## Highlights

► Geographical metrics including land use and landscape patterns were assessed. ► Contrasted levels of nitrogen and pesticides were detected in the seven hydrosystems. ► Proteomics sharply reflected metabolic alterations of fish submitted to multistress. ► Fish showed detoxification, hypoxia response, alterations of urea cycle and immunity. ► The major environmental stressors were identified within each hydrosystem.

**Keywords** : Land use, Estuarine water quality, Hypoxia, Pollutants, Shotgun proteomics, Platichthys flesus

## 1. Introduction

Estuarine environments are commonly exposed to multiple stressors and are therefore considered as very sensitive systems (Bárcena et al. 2017; Elliott et al. 2014). Stress factors mainly originate from the catchment area, and are attached to agriculture, industry, demography, or urbanization (Elliott, 2011), as well as global change (Robins et al. 2016). The combination of these multiple pressures will influence the quality of estuaries, resulting in a loss of biodiversity and a general decrease in the quality of transitional waters linked to eutrophication and pollution (Elliott, 2011). Among sensitive estuarine ecosystems, small-sized ones are mainly affected by water quality degradation due to human activities rather than structural developments, and anthropization is assessed by measuring the impact of both eutrophication and pollution. In addition, small estuaries exhibit limited environmental heterogeneity, making it easier to compare biotic responses with reduced within basin inter-individual variability (Doubleday et al. 2015; Vasconcelos et al. 2015; Elliott, 2011).

In France, agricultural land accounts for 45 % of the total surface area, and can even reach over 60 % in certain regions, such as Brittany (Insee, 2021). Moreover, France is the leading producer of agricultural products in the European Union, with 77 billion euros in 2019 (18 % of the European total production, Eurostat). Given the importance of agriculture to the national economy, France is one of the leading importers and consumers of pesticides (insecticides, herbicides and fungicides) in Europe (ranked 1$^{st}$) and in the world (ranked 7$^{th}$) (FAO, 2024). Pesticides are transported from urban and agricultural areas to estuaries through the river system and by stormwater (Topaz et al. 2018; Carpenter et al. 2016). The toxicity of these products to aquatic ecosystems can induce habitat deterioration (Echeverría-Sáenz et al. 2012), the loss of sensitive species (Beketov et al. 2013), and even significant mortality (Bille et al. 2017). Thus, pesticides have clearly been identified as the main source of surface and groundwater contamination and have become a major environmental concern in Europe (Leistra & Boesten, 1989). However, the impact of pesticides on small estuaries has been poorly studied by researchers (Callaway et al. 2014), whereas these systems (coastal rivers) are very common in France, and even more so in Brittany. Furthermore, these small systems are of major interest to many municipalities, as they constitute water catchment areas for drinking water production.

The European flounder (*Platichthys flesus*) is an estuarine resident species living principally in the oligo and mesohaline areas which are displaying the most stressful estuarine conditions (Teichert et al. 2017); this fish being considered as a very relevant species for the assessment of the water quality of estuarine systems (Williams et al. 2011; Borcier et al. 2020). Thus, in a first survey (Laurent et al. 2023) conducted in five moderately-sized estuaries in Brittany (France), we integrated environmental signatures and omics-approaches on juvenile European Flounder, and showed contrasted ecological status between hydrosystems. In the present study, we go further by studying six new estuaries and their hydrosystems located in Brittany,

whose total catchment area were between 70 and 450 km², and by comparing them to our previous reference hydrosystem lowly impacted by stressors (Laurent et al. 2023).

The originality of the present survey resides in the study of seven hydrosystems mainly impacted by agricultural activities (production of cereals, vegetables and livestock; fish farming). In addition, four of these watersheds are adjacent, and this geographical proximity could limit the detection of a possible differentiation of their health status. Furthermore, one of the studied system is fragmented by two dams downstream, the last huge dam (20 m high and 102 m long) being located immediately upstream the estuary. We therefore investigated whether our multidisciplinary approach is sufficiently powerful to differentiate these systems, which appear at first sight to be rather similar. Moreover, we kept the previous reference hydrosystem in this comparison to conduct a temporal study and confirm, or not, its relatively good ecological status highlighted previously (Laurent et al. 2023).

The first objective of this study was to establish an accurate typology of these small hydrosystems, to identify their potential stressors and confirm, or not, the reference status of the Aven estuary. To this end, we compared different environmental parameters measured over the seven watersheds from landscape (anthropogenic land cover, hydrobiology) to estuary (sediment quality, chemical contamination), and fish individual scales (environmental signatures).

The second objective focused on the analysis of the liver molecular responses of the European Flounder to stressors. Omics approaches accurately reflect the overall metabolic alterations of organisms submitted to multi-stress environment ; they are increasingly considered in the context of biomonitoring (Laurent et al. 2023; Borcier et al. 2019; Jeffrey et al. 2019, Gouveia et al. 2019; Martyniuk, 2018; Bahamonde et al. 2016; ECETOC, 2010). Thus, we conducted shotgun proteomics on flounders collected in the seven estuaries, comparing the differential proteins accumulation in fish liver between sites. Then, the combining of environmental signatures and proteomic data should allow to identify the major stressors per hydrosystem. We hypothesised that shotgun proteomics performed at fish individual scale would sharpen our integrative approach: (1) to differentiate *a priori* similar hydrosystems, and (2) to better qualify the health status of estuaries.

## 2. Materials and methods

### 2.1. Study sites and landscape scale metrics

Seven hydrosystems were covered, from the watershed to the estuary: Gouessant, Guillec, Flèche, Quillimadec, Aber Wrac'h, Douffine and Aven. These systems are located in Brittany, along the French Western Channel coast and the French Atlantic coast (Fig. 1).

Ten geographic indicators were developed from different national and regional geographic databases (Table 1). In addition to the size of the catchment area (in km2), these metrics were used to produce a first typology of anthropogenic activities (agriculture, urbanisation) and of the ecological status of the different

watersheds. Three indicators concerned agriculture and associated pressures. One of these metrics measures the all-feed livestock unit index in the watersheds. Provided by the Agreste database of the French Ministry of Agriculture, this index measures food rations consumed by the different species of livestock. In Brittany, the livestock consists mainly of cattle, pigs and poultry. The index is calibrated on the grazing equivalent of a 600 kg dairy cow producing 3,000 kg of milk per year. The proportions of agricultural land in the watersheds were also measured using the Graphic Land Register (RPG). The quantity of pesticides applied on agricultural surfaces was assessed using the treatment frequency index (IFT), provided by Agreste (Crisan, 2020; Pujol, 2015). This index provides information on the number of doses of pesticides treatments (insecticides, herbicides and fungicides) - i.e. spraying crops with chemicals to reduce the development of diseases and pests - applied per hectare. A dose is the recommended amount to be applied during a spraying. Indicators related to urbanization have also been developed. Urban pressure is assessed using the INSEE (French National Institute for Statistics and Economic Studies) data base to calculate population density. Similarly, the 2019 Theia-Land data (10 m spatial resolution), from the DataTerra research infrastructure, were used to calculate the proportion of artificial surfaces per watershed. Three indicators of the good ecological status of the watershed were developed from the Theia-Land and BD TOPO databases (from IGN – French national mapping agency, at 1:10 000 scale) to calculate the percentage of natural surfaces (such as forest), the density of hedges and the percentage of riparian vegetation. Riparian vegetation is that which occupies the channels and banks of streams (Aguiar et al. 2011), while a hedge is made up of shrub and tree vegetation organized in a linear fashion (ERB). In Brittany, hedgerows delimit meadows and cultivated fields of various sizes and shapes, making up a landscape mosaic known as the "bocage" (OFB). The higher the density of hedgerows, the more complete the bocage is considered to be, and the more likely it is to help control the flow of water, soil erosion and the spread of pollutants (Boinot et al. 2023). Riparian vegetation is able to play the same role, but in the immediate vicinity of streams. The percentage of riparian vegetation expresses its potential role as a buffer zone between urban and agricultural areas and streams.

### 2.2. Environmental fish sampling and tissue collection

Within each estuary, we sampled juvenile European flounders (young of the year) that were born in March in coastal areas and recruited in May in the estuaries. These immature fish therefore spent approximately 5 months in summer estuarine conditions before their catch by electrofishing in the upstream part of the estuaries, between mid-September and mid-October 2020. Twenty flounders (average total length: 9.16 ± 1.14 cm) were caught per estuary. Each individual was weighed and measured to determine the fish condition index ($K = 100 \times (W/L^3)$, where W is the weight of the fish in g and L the total length in cm). Then, each individual was sacrificed in the field and dissected to collect several tissues (liver and muscle). Recovered livers were immediately flash frozen in liquid nitrogen and stored at -80°C. Otoliths were also collected, stripped of their membranous labyrinths and preserved in dry Eppendorf tubes.

### 2.3. Chemical analyses and environmental signatures at estuary and fish individual scales

142 2.3.1. Analyses of dissolved inorganic nitrogen (DIN) in estuarine waters

143 Nitrate and nitrite concentrations were assessed using the methodology detailed by Aminot & Kérouel (2007)
144 as in Laurent et al. (2023). To this end, water samples were collected in the upper estuaries at low tide, just
145 before fish sampling. Approximately 15 mL of water was filtered (0.2 µm) and frozen at -20°C until analysis.
146 The samples were then analysed using a Bran + Luebbe AAIII auto-analyser. The concentrations of DIN in
147 water collected before the fish sampling were compared with the annual average concentrations assessed
148 by DREAL (Regional Directorate for the Environment, Development and Housing - Rennes) in the
149 hydrosystems in 2019, excepted for the Aven in 2016.

150 2.3.2. Pesticides in the downstream part of watersheds

151 As part of surface water quality monitoring, French local authorities and water managers collected water
152 samples that have been transferred to laboratories carrying out analyses of organic pollutants in water.
153 Pesticide analyses were performed on freshwater samples collected after rainy weather (>10 mm over the
154 last 24 hours). All samples contained at least one active substance at a concentration greater than 0.1 $\mu g.L^{-1}$,
155 and over 150 molecules were analysed by liquid and gas chromatography coupled with tandem mass
156 spectrometry (LC-MS/MS and GC-MS/MS). Each water manager provided us the reports detailing for 2018-
157 2019: average cumulative total pesticide concentrations in water, detection frequencies of the main
158 molecules, or the raw data.

159 2.3.3. Organic pollutants in estuarine sediment and fish tissue

160 Organic pollutants were quantified in sediment and flounder tissue by sorptive stir bar extraction-thermal
161 desorption-gas chromatography-tandem mass spectrometry (SBSE-GC-MS/MS) using a method adapted
162 from Lacroix et al. (2014) and performed in Laurent et al. (2022; 2023). In total, the concentrations of 7 PBDEs,
163 24 PAHs, and 26 PCBs were measured. Briefly, 100 mg of sample (w.w.) was digested by saponification. Then,
164 analytes were extracted for 16 h at 700 rpm using polydimethylsiloxane bars (Twister 20 mm x 0.5 mm,
165 Gerstel). The bars were then analysed using an Agilent 7890A gas chromatography system coupled to an
166 Agilent 7000 triple quadrupole mass spectrometer (Agilent Technologies) and equipped with a thermal
167 desorption unit (TDU) combined with a cooled injection system (Gerstel). The GC column was a Restek Rxi-
168 5ms (30 m, 0.25 mm, 0.25 µm). Analytes were evaluated against deuterated compounds using a calibration
169 curve ranging from 0.01 ng to 30 ng per bar. Samples were dried at 50°C until the mass remained constant
170 to remove water from the tissue. Results are therefore expressed as µg of analytes/kg dry weight (d.w.).
171 Limits of quantification (LOQs) were calculated by the calibration curve method (Shrivastava & Gupta, 2011)
172 and limits of detection (LODs) were estimated by dividing the LOQ by three. Analytical quality control was
173 performed using the 1974c standard reference materials "Organics in Mussel Tissue (*Mytilus edulis*)"
174 provided by the National Institute of Standards (SRM) and Technology (NIST, Gaithersburg, USA).

2.3.4. Trace elements in estuarine sediment and fish muscle

Two sediment samples and ten fish muscle samples per studied estuary were analysed as presented in Laurent et al. (2023). Muscle samples were lyophilized (Alpha 1-2 LDPlus, CHRIST) for 48h. All samples were oven-dried for 12h at 60°C before being ground in an agate mortar. Then, approximately 50 mg of powdered muscle and sediment (weighted with a precision of 0.01 mg) were mineralized in closed 15-mL Teflon screw-cap vials (Savillex®) with 1 mL of 65 % nitric acid (Merck, Suprapur®) and 250 µL of 30 % suprapure hydrogen peroxide (Merck, Suprapur®). The muscle and sediment samples were then hydrolysed for 3h at 85°C and 4h at 105°C (EasyDigest®, ANALAB), respectively. Diluted mixtures (2.5 % nitric acid) were used for measurements of As and of several trace metals including Cd, Co, Cr, Cu, Mn, Pb, V and Zn, using an ICP-quadrupole mass spectrometer (X-series II, Thermo Scientific) operated at Pôle Spectrométrie Océan Brest (PSO, Brest, France). The concentrations reported in this survey were above the limits of quantification, while the digestion blanks were below the limits of detection. Certified reference material (DORM-4 fish protein, National Research Council of Canada) was used to assess analytical accuracy.

2.3.5. Trace elements analysis in fish otoliths

Trace element analyses performed on whole otoliths provides an average signature over the entire life of the fish. Otoliths from ten fish per estuary were used for trace element analysis in the same method as in Laurent et al. (2023). Briefly, otoliths were cleaned, rinsed and stored with milliQ water in an Eppendorf tube, previously treated with 10 % nitric acid. The otoliths were then exposed to an ultrasonic bath for 5 min and dried in a fume hood for 48h. Each otolith was weighed (1.92 ± 0.44 mg) using a Mettler Toledo MX5 balance and transferred to a Teflon tube. To each Teflon tube containing the samples, 2 mL of 2.5 % ultrapure nitric acid spiked with 0.863810 ppb indium (internal standard of the samples) was added in the clean room. The exact amount of acid added was measured on an accurate balance to 0.1 mg (1.21 ± 0.008 g). An internal standard, indium, and a standard sample were used to correct for signal drift and to verify the accuracy of the method during ICP-MS analysis. For this, 11.33 mg of northern red snapper (*Lutjanus campechanus*) sagittal otolith powder was added to 22.24 g of nitric acid. Elemental analysis was performed using a sector field ICP-MS (Thermo Element XR), in low and medium resolution depending on the selected isotopes. In low resolution, were measured $^{7}$Li, $^{11}$B, $^{97}$Mo, $^{111}$Cd, $^{118}$Sn, $^{121}$Sb, $^{135}$Ba, $^{138}$Ba, $^{208}$Pb and $^{238}$U. In average resolution were investigated $^{25}$Mg, $^{51}$V, $^{52}$Cr, $^{55}$Mn, $^{56}$Fe, $^{59}$Co, $^{60}$Ni, $^{63}$Cu, $^{66}$Zn, $^{86}$Sr, $^{138}$Ba and $^{75}$As. Limits of quantification were calculated for each of the analysed isotopes based on 10 times the standard deviation of the blanks.

2.3.6. Stable isotopes analysis in fish otoliths

Stable oxygen and carbon isotope ratios were determined from otoliths of ten flounders per studied sites as performed by Laurent et al. (2023). Otoliths were cleaned, rinsed, and ground. Then, approximately 100 µg of powdered otoliths (111.3 ± 26.8 µg) were weighed and placed in a specific glass vial for isotopic analyses. Isotopic analyses were performed using a MAT-253 stable isotope ratio mass spectrometer (Thermo

Scientific) coupled to a KIEL IV carbonate apparatus (Thermo Scientific). The standard deviation was calculated using a homogeneous internal carbonate standard. It was ± 0.03 ‰ for $\delta^{18}O$ and ± 0.02 ‰ for $\delta^{13}C$ for this mass range (50-170 µg). To formulate the V-PDB scale values, all samples were calibrated using two international carbonate standards, NBS-19 ($\delta^{18}O$=-2.20 ‰ and $\delta^{13}C$=+ 1.95 ‰) and NBS-18 ($\delta^{18}O$=-23.20 ‰ and $\delta^{13}C$=-5.01 ‰).

### 2.4. Shotgun proteomics at fish individual scale

#### 2.4.1. Total protein extraction, trypsinolysis and peptides analysis by tandem mass spectrometry

The complete proteome of five fish livers per estuary were analysed, as in Laurent et al. (2023). Briefly, protein extracts were diluted in 30 µL of NuPAGE LDS 1X sample buffer (Invitrogen) and 20 µL of β-mercaptoethanol. Based on its protein concentration, each sample was diluted with water and NuPAGE LDS 3X (Invitrogen) to a total protein concentration of 1 $\mu g.\mu L^{-1}$ in LDS 1X. The samples were then heated at 99°C for 5 min. For each sample, 30 µg of protein was plated on a NuPAGE 4-12 % gradient gel and subjected to SDS-PAGE in MES SDS migration buffer (50 $mmol.L^{-1}$ MES ([2-(N-morpholino) ethane sulfonic acid), 50 $mmol.L^{-1}$ Tris Base, 0.1 % SDS, 1 $mmol.L^{-1}$ EDTA, pH 7.3) for 5 min. The proteome of each sample was extracted as a single polyacrylamide strip and processed for trypsin proteolysis as described in Hartmann et al. (2014). A 50 µL peptide sample was thus obtained. One-fifth of the volume of this sample was injected into a C18 PepMapTM 100 nanoscale capillary column (LC Packings) and resolved with a 120-min gradient of $CH_3CN$, 0.1 % formic acid, at a flow rate of 0.2 $\mu L.min^{-1}$. Data-driven acquisition analysis of the peptides eluting from the column was performed with a Q-Exactive HF (Thermo) mass spectrometer (Klein et al. 2016). Each full scan of peptide ions in the Orbitrap analyzer was acquired from m/z 350 to 1800 at a resolution of 60,000 and with a dynamic exclusion of 10 seconds. Each MS scan was followed by high-energy collision dissociation and MS/MS scans on the 20 most abundant precursor ions.

#### 2.4.2. Proteomic data interpretation

MS/MS spectra were assigned to peptide sequences by the MASCOT Daemon 2.3.2 search engine (Matrix Science) using the complete annotated proteomic database of *Paralichthys olivaceus*, as described previously in Laurent et al. (2023). The abundance of proteins in each condition was assessed by their spectral count (number of MS/MS spectra per protein). A protein was validated and conserved when at least two different peptides (p-value less than 0.05) were detected. The false discovery rate of protein identification is thus less than 1 %, as verified with a reverse search of the decoy database.

#### 2.4.3. Proteomics data deposition

The mass spectrometry and proteomics dataset are available through the ProteomeXchange Consortium via the PRIDE partner repository (https://www.ebi.ac.uk/pride/), under dataset identifiers PXD045611 and

10.6019/PXD045611. [The reviewers may access the currently private dataset using reviewer_pxd045611@ebi.ac.uk as Username and xLByP0vpas Password].

#### 2.4.4. Functional analysis

For each estuary, proteomic dataset was subjected to a functional analysis involving a comprehensive COG (Cluster of orthologous group) classification coupled with GO (Gene Ontology) and KEGG enrichment experiments, as well as molecular network analysis using STRING v11.0 (Szklarczyk et al. 2019). These approaches aim to identify differentially regulated metabolic pathways in the different estuaries. To identify the primary metabolic shifts in the livers of fish collected from different estuaries, we conducted a functional proteome analysis. In this context, we retrieved and re-annotated the sequences of all dysregulated proteins using Blast2GO and EggNOG (Laurent et al. 2023; Powell et al. 2014; Conesa et al. 2005), followed by a detailed manual re-examination.

### 2.5. Statistical analysis

Statistical tests were performed in R. Normality and homoscedasticity of variances were investigated with a Shapiro-Wilk test and a Bartlett test, respectively. Because the data were not normally distributed, a nonparametric Kruskal-Wallis test followed by a Dunn's post hoc test (for multiple comparisons) were applied to compare the means. A p-value below 0.05 was assumed as a significant difference. Data integration was conducted by principal component analyses (PCA) using the FactorMineR software package with default settings.

## 3. Results & Discussion

### 3.1. Land use in watersheds

The geographical indicators developed in this study, focused on seven watersheds located in the Brittany region (France), and highlighted the main characteristics and anthropogenic activities of these moderate-sized hydrosystems (73 < catchment area ($km^2$) < 420). Land use and occupation, determined by a geographic information system (GIS), enabled the systems to be segregated by groups (Table 1).

A first group of watershed (Gouessant, Guillec, Flèche, Quillimadec, Aber Wrac'h) displayed a higher agricultural pressure (agricultural surface ≈ 75 %, and 285 $UGBTA.ha^{-1}$ < livestock < 534 $UGBTA.ha^{-1}$) compared to a second group (Douffine and Aven) showing less marked agricultural surfaces (60 - 70 %) and livestock (190 - 220 $UGBTA.ha^{-1}$) (Table 1). Pesticide treatment frequencies were also higher in the first group (2.4 < IFT. $Catchment^{-1}$ < 3.8) compared to the second group (1.8 < IFT. $Catchment^{-1}$ < 2.1) (Table 1).

The fish farm production showed the highest production of trout in Douffine (900 $T.year^{-1}$), followed by a significant production of ≈ 400 $T.year^{-1}$ in Guillec and Aven (Table 1).

Information on population density and urbanization levels in the seven watersheds studied was also gathered (Table 1). The highest population densities were reported for Aber Wrac'h and Quillimadec (118 - 172 inhab.km$^{-2}$). The population density being moderate in the other watersheds (31 - 85 inhab.km$^{-2}$). The relatively high population densities were associated with the highest occurrences of urbanized areas, observed for Aber Wrac'h (18.7 % of catchment) and Quillimadec (19.1 % of catchment). The lowest occurrence of urbanized areas was detected in the Douffine watershed (5.6 % of catchment).

Lastly, several geographic ecological quality metrics were assessed in the watersheds (Table 1). These metrics included natural areas, hedgerow density and riparian vegetation. Aven and Douffine showed the highest rates of preserved natural areas (19 - 35 %) and the highest rates of riparian vegetation (25 - 33 % of the 100 m river band); the prevalence of riparian vegetation enhancing water quality (Chua et al. 2019; Dosskey et al. 2010) and supporting ecological corridors (Gregory et al. 2021). The two previous watersheds contrasted sharply with the group: Guillec, Flèche, Quillimadec, Aber Wrac'h which displayed reduced natural surfaces (8 - 10 % of catchment) and limited riparian vegetation (13 - 18 % of the 100 m river band). The hedge density metric did not differentiate the majority of watersheds (7500 m.km$^{-2}$ - 9800 m.km$^{-2}$) but showed a particularly weak value for the Gouessant basin (5600 m.km$^{-2}$) mainly characterized by a very high pressure of cereal crops.

### 3.2. Chemical parameters and environmental signatures

To assess water quality, we analysed the main pollutants associated with human activities: nitrates and nitrites in freshwater related to nitrogen fertilizers and wastewater, and pesticides used in agriculture and detected in freshwater. Furthermore, several xenobiotics were also analysed in sediment and fish, related to road traffic and domestic and industrial effluents (PAHs, PCBs and metals). We also measured trace elements and stable isotopes in fish otoliths, to better characterize: exposure to pollutants, fish habitat and fish metabolic rate (Laurent et al. 2023).

#### ***Dissolved inorganic nitrogen (DIN) in water***

DIN annual average concentrations (monthly basis) assessed by DREAL in 2016 (Aven) and 2019 (other hydrosystems) were very similar to our analyses on water collected in the field before fish sampling (Table 2). Regarding nitrite (Table 2), the highest concentrations were observed in the group composed of Gouessant, Guillec and Douffine (0.12 - 0.16 mg.L$^{-1}$). The other estuaries showed reduced levels of nitrite (≈ 0.05 mg.L$^{-1}$). The highest nitrate concentration was observed in Guillec (50 mg.L$^{-1}$); medium values being observed in the group consisting of Flèche, Quillimadec and Aber Wrac'h (≈ 33 mg.L$^{-1}$). Finally the level of ammonium was higher in Guillec (0.4 mg.L$^{-1}$), the other estuaries showing an average concentration of 0.1 mg.L$^{-1}$; Aven displaying the lowest ammonium level (0.03 mg.L$^{-1}$).

The high loads of DIN ($NH_4^+$, $NO_2^-$, $NO_3^-$) (Table 2), particularly detected in Guillec, could be linked to a strong fertilisation in small parcels devoted to intensive vegetable production (potatoes, carrots, shallots). The

306 group composed of Flèche, Quillimadec, and Aber Wrac'h showed high loads of $NO_3^-$ that could be also
307 related to the agricultural pressure. On the other hand, Aven displayed a reduced load of DIN; this trend was
308 also detected in a previous study (Laurent et al. 2023). This last result confirmed that the agricultural pressure
309 is quite moderate in Aven; this watershed is mainly oriented towards the production of canned vegetables
310 and shows marks of a good conservation of the bocage that could reduce the fluxes of nitrogen over the
311 system.

312 ***Pesticides in freshwater***

313 Several campaigns were conducted by the hydrosystem managers in the downstream part of the rivers, to
314 collect surface freshwater samples after rainy events, over the period 2018-2019. Then, pesticide analyses
315 were carried out; results being compiled in water quality database which were subsequently communicated
316 to us by the managers. Thus, we have assessed the average total cumulative pesticide concentration in water
317 over the year, and the detection frequencies of the main molecules, per hydrosystem (Table 3).
318 The annual mean of cumulative total pesticide concentrations showed higher values in Gouessant and Guillec
319 (Total Pesticide Concentration: TPC ≈ 6.50 $\mu g.L^{-1}$) *vs* the other hydrosystems (1.16 ≤ TPC≤ 2.12 $\mu g.L^{-1}$),
320 probably related to their intensive production of cereals and vegetables. This level of water pesticide
321 concentration was classically observed in a little catchment (200 $km^2$) dedicated to agriculture in the South-
322 West France, with TPC in River Trec = 1.6 $\mu g.L^{-1}$ (Poulier et al. 2014). In the present study, the lowest values
323 of water pesticides were detected in Quillimadec, Aven and Douffine (1.25 ≤ TPC≤ 1.45 $\mu g.L^{-1}$); hydrosystems
324 showing a less intensive agriculture, and for the Douffine a reduced percent cultivated crops in the watershed
325 (Table 3).
326 The frequencies of major detected molecules (Table 3) showed a general contamination of water surface by
327 metabolites of Metazachlor (ESA and/or OXA) and Metolachlor (ESA and /or OXA) in the whole hydrosystems.
328 These herbicides are widely used in intensive agriculture in Europe (Slaby et al. 2023; Bernard et al. 2019;
329 Weber at al. 2018; Poulier et al. 2014; Boithias et al. 2011) and in U.S. (Van Metre et al. 2017); Metazachlor
330 and Metolachlor being principally used in corn, wheat, barley and rape rotations. The herbicide Acetochlor
331 was particularly frequent in Guillec, Flèche, Quillimadec and Aven (Table 3) for corn treatment. Furthermore,
332 other herbicides were also commune in several watershed (2,6 dichloro-benzamide; Glyphosate and its
333 metabolite AMPA; Atrazine; ASDM), and widely used in cereals and vegetables productions. The Chlorprofam
334 was specifically detected in the Gouessant; the treatment by this herbicide is carried out in shallot, onion and
335 potato productions.
336 The higher diversity of pesticides was clearly detected in the intensive agriculture characterizing Gouessant
337 and Guillec, with the presence of specific fungicides (Table 3). Propiconazole and Tebuconazole were
338 frequent in the Gouessant and mainly used in cereal production; these molecules being also widely
339 distributed in Midwest U.S. (Van Metre et al. 2017). On the other hand, Boscalid, Dimetomorph and Oxadixyl

were fungicides observed in the Guillec and principally used in vegetables production. Among the detected fungicides in the present study, Boscalid and Tebuconazole are frequently used in Europe (Le Cor et al. 2021).

***Organic pollutants in estuarine sediment and fish tissue***

The analyses of PCBs in the sediment remained below the detection limits in the seven estuaries which highlighted a limited impact of industrial effluents in these mainly agricultural watersheds (Table 2). The fish muscle PCBs concentrations were particularly low (0.7 - 28 $ng.g^{-1}$ d.w.) among all the studied agricultural watersheds; these values being very weak compared to those commonly observed in the flounder muscle collected in the industrial estuaries in France (Seine estuary ≈ 470 $ng.g^{-1}$ d.w. in Borcier et al. 2020) or in England (Mersey estuary ≈ 1580 $ng.g^{-1}$ d.w. in Williams et al. 2011). The highest PAHs concentrations in sediment were observed in Aven (1480 $ng.g^{-1}$ d.w.), due to the presence of a marina at the sampling site and a car park very popular for tourists in summer. Medium values of PAHs in the sediment (270 - 530 $ng.g^{-1}$ d.w.) were observed for the group composed of Flèche, Quillimadec, Aber Wrac'h and Douffine; they are also probably linked to road traffic over these watersheds. Reduced levels of sediment PAHs were detected in Gouessant and Guillec (≈ 110 $ng.g^{-1}$ d.w.). Globally, the contamination of sediments by PAHs appears moderate in the seven studied agricultural basin compared to the level observed in French industrial estuaries (upstream Seine estuary ≈ 7200 $ng.g^{-1}$ d.w. in Laurent et al. 2024).

***Trace elements in estuarine sediment and fish muscle***

Sediment analyses (Table 2) highlighted a pronounced multi-contamination in the Douffine estuary, especially in As (14 $\mu g.g^{-1}$ d.w.), Cd (2 $\mu g.g^{-1}$ d.w.), Cu (69 $\mu g.g^{-1}$ d.w.), Pb (261 $\mu g.g^{-1}$ d.w.) and Zn (326 $\mu g.g^{-1}$ d.w.); this metallic multi-contamination being linked to ancient mining activities in a related watershed (Chiffoleau, 2017). Lower concentrations of trace elements were generally detected in Aven and Aber Wrac'h sediments, with the exception of As (27 $\mu g.g^{-1}$ d.w. in Aber Wrac'h and 13 $\mu g.g^{-1}$ d.w. in Aven). Globally, the remaining watersheds (Gouessant, Guillec, Flèche and Quillimadec) displayed low contaminations of sediments by trace elements.

The determination of trace elements in the muscle of the flounder caught in Douffine did not reflect the multi-contamination observed in the sediments (Table 2). The relationship between trace elements in sediment *vs* fish appeared weak, with the exception of the highest As concentrations also measured in Aven (6 $\mu g.g^{-1}$ d.w.) and Aber Wrac'h (3 $\mu g.g^{-1}$ d.w.). Globally the differentiation of fish trace elements between watersheds was reduced, the Aven basin showing the lowest values of Cd, Cu, Pb and Zn in fish muscle.

***Trace elements and isotopes in fish otolith***

Trace element and stable isotope compositions were analysed for 10 flounder per system studied (Table 2 & Fig. 2) at the scale of the entire otoliths integrating the lifetime of the fish, from its birth to its capture. Otoliths can contain many trace elements such as Li, Sr, Ba or Mn. Rare earth elements (REE) and elements

373 such as Hg or Pb were also found in otoliths (Hüssy et al. 2021). All these trace elements are used to
374 reconstruct fish environmental changes and to detect possible exposure to pollutants (Halden & Friedrich,
375 2008).
376 Trace element analyses in otoliths showed that two major elements can characterize the fish habitats in
377 estuaries, *i.e*. Sr and Mn. The Sr:Ca ratio in otoliths is commonly used as a proxy for water salinity and
378 migration patterns (Panfili et al. 2015; Zimmerman, 2011, 2005), regardless of diet (Lin et al. 2007). In the
379 present study, the highest Sr concentrations (Table 2) in otoliths were clearly measured in Aven (1655
380 µmol.mol$^{-1}$ Ca) suggesting a flounder polyhaline environment. The lowest Sr levels were measured in the
381 group composed of Gouessant, Guillec, Flèche and Douffine (423 - 650 µmol.mol$^{-1}$ Ca), in relation to
382 oligohaline environments for fish. The medium values for otolith Sr in Quillimadec and Aber Wrac'h (811-
383 1154 µmol.mol$^{-1}$ Ca) being linked to a mesohaline environment.
384 The Mn:Ca ratio in fish otoliths, which could be sensitive to growth effects (Limburg & Casini, 2018), is
385 commonly used as a proxy for hypoxia in water bodies (Jiang et al. 2022; Limburg & Casini, 2019; Limburg et
386 al. 2015; Thorrold & Shuttleworth, 2011). Thus we suggest that the highest Mn concentrations (Table 2)
387 observed in Gouessant (36 µmol.mol$^{-1}$ Ca) and Douffine (30 µmol.mol$^{-1}$ Ca) revealed hypoxic events in these
388 two estuaries.
389 Isotopic analysis were carried out on $\delta^{18}O$ and $\delta^{13}C$ otoliths compositions. The fish otolith $\delta^{18}O$ composition
390 was commonly used as a proxy of water salinity, but can also be used to reconstruct water temperature (Reis-
391 Santos et al. 2023). The otolith $\delta^{13}C$ composition is now considered as a very relevant proxy of the field
392 metabolic rate of teleost fishes (Chung et al. 2019). The enriched $\delta^{18}O$ values for Aven (Fig. 2) confirmed the
393 polyhaline environment detected previously by the Sr concentrations in this particular system. The other
394 estuaries were located in the left part of the diagram, and thus characterized by depleted $\delta^{18}O$ values linked
395 to meso- and oligohaline environments, and also probably to higher water temperatures. Considering the
396 $\delta^{13}C$ otolith composition (Fig. 2), we suggest that the enriched values for the Aven flounders in the right part
397 of the diagram could be linked to a reduced fish metabolic rate, in a weakly stressed environment. The most
398 depleted compositions on the left part of the diagram (Fig. 2), particularly those between -16 and -14 for the
399 Flèche estuary, could be linked to an increase of the fish metabolic rate in a highly stressed environment.
400 This activation of the metabolic rate allows to respond to the increasing energy cost of self-maintenance in
401 stressed environments (Sokolova, 2013).

402 ***Fish condition index***

403 The fish condition index showed no significant differences between the fish populations in the seven
404 estuaries (Table S1). Thus we have not detected an apparent loss of fish fitness in particular systems over the
405 whole studied watersheds.

406 3.3. Integration of environmental signatures and identification of the reference hydrosystem

A principal component analysis was conducted on the environmental signatures, integrating: geography of the watersheds, hydrobiology, chemical data and fish environmental signatures (Fig. 3). The first two PCA axes represented 43.4 % of the total variance. The axis one (horizontal) separated a first group of watersheds (Gouessant, Guillec, Flèche, Aber Wrac'h and Quillimadec) on the left part characterized by high agricultural pressures (high levels of: agricultural surface, pesticide treatment, water nitrate) from the Douffine basin on the right part showing a moderate agricultural pressure (high levels of natural surfaces and riparian vegetation) but high metals concentrations in the sediment and important fish farm activities. Furthermore, within the first highly anthropised group, Guillec and Gouessant showed high freshwater pesticides, overload of nitrites and a fish metallic contamination by Cu and Zn, whereas Quillimadec and Aber Wrac'h displayed high population density and urbanised surface.

The axis two (vertical) of the Figure 3 highlighted in the lower part of the diagram, high Mn otolith values shared in Gouessant and Douffine indicating hypoxic events in these hydrosystems. The Aven watershed was isolated in the upper side of the diagram in relation with the absence of hypoxic events, and reduced level of water pesticides. Furthermore, this system was mainly characterized by a polyhaline environment (high Sr otolith), high hedge density, reduced trace elements contamination in fish and sediment, with the exception of an As signal in fish muscle. Thus, no major stressors were detected in the Aven basin, and fish displayed a reduced metabolic activity in the Aven estuary (high otolith $\delta^{13}C$), highlighting a less stressed system compared to the six other watersheds. Therefore, we have chosen the Aven watershed as the reference system in the proteomics analysis in the following sections.

### 3.4. Shotgun proteomics

The global shotgun proteomics approach developed here aimed to identify differences in the liver metabolism of flounders sampled from various estuaries. The liver is a highly active organ in terms of metabolism and serves as the primary detoxification centre for fish. The observed differences between the livers collected in the reference estuary (Aven) compared to liver from the six tested estuaries may, therefore, reflect the multi-stress experienced by the fish in their habitats. The fish health thus mirrors the ecosystem health.

In total, we analysed 726,163 MS/MS spectra, which allowed us to identify and track 1,740 proteins across all analysed samples. Comparative statistical analysis was conducted using PatternLab software. Additionally, as previously mentioned, we used the Aven estuary as a reference point. The significance level was set at a p-value of 0.05, and the fold change factor (Tfold) was set at 1.5.

Proteomic comparisons revealed that the smallest differences in fish liver proteomes were observed between Guillec *vs* Aven, followed by Aber Wrac'h *vs* Aven (Table 4). Specifically, for individuals from Guillec, the abundances of 44 proteins increased, while 45 ones decreased. Similarly, 33 proteins were up-regulated, and 60 proteins were down-regulated in the livers of flounders from Aber Wrac'h. In contrast, the most

441 significant differences were found in fish from Gouessant, with 202 proteins showing increased abundance
442 and 46 proteins displaying decreased abundance (Table 4).
443 Determining whether the number of dysregulated proteins between the studied estuaries is proportional to
444 the intensity of stress on the organisms is challenging. Our study did not include an unstressed control
445 environment since we were investigating individuals collected from their natural habitats. This implies that
446 even the fish from the reference estuary may have been potentially exposed to some particular stressors.
447 Therefore, we supplemented the study with a qualitative and quantitative functional analysis of differentially
448 accumulated proteins to gain more meaningful insights.

449 ***Functional re-annotation and COG classification***

450 In response to changing environmental conditions, organisms continually adjust their functioning by
451 undergoing metabolic modifications. This functional analysis was based on the classification of different
452 protein subsets using COGs (Clusters of Orthologous Groups) (Fig. S1). This initial analysis revealed a
453 remarkable functional differences within the proteome between estuaries.
454 In the Gouessant estuary, we observed the prevalence of categories O (16.3%, protein
455 modification/transformation) and J (11.4%, translation, ribosomal structure, and biogenesis) in up-
456 regulation, and category T (10.4%, signal transduction) in down-regulation.

457 In fish sampled from Guillec, three enriched protein categories (A, G, T) were identified at 16.6% (A for RNA
458 processing and modification, and G for carbohydrate transport and metabolism). Down-regulated proteins
459 were predominantly associated with categories O (17.8%) and S (15.6%).
460 In the Flèche estuary, category O was the most dysregulated, with 18.9% and 18.6% of the accumulated and
461 reduced proteins, respectively. Categories G, E (amino acid transport and metabolism), and I (lipid transport
462 and metabolism) were also enriched, with 15.5%, 13.8%, and 10.3%, respectively.
463 In Quillimadec, we observed the dominance of categories J (22.7%) in accumulated proteins and S (24.6%,
464 function unknown) in decreased ones. Categories U (22.7%, intracellular trafficking, secretion, and vesicular
465 transport) and O (12.7%) were also up-regulated, while category Q (14.8%, secondary metabolites
466 biosynthesis, transport, and catabolism) was downregulated.
467 In Aber Wrac'h, categories C (energy production and conversion), E, O, and Q largely dominated, each at
468 12.1%. Category I was also up-regulated with 15.2%. Downregulated proteins were mainly associated with
469 categories O (13.3%), Z (13.3%, Cytoskeleton), and E (10%).
470 Finally, in Douffine, the most over-accumulated proteins were in categories I (12.7%), O (12.7%), and T
471 (11.3%). Conversely, the most under-accumulated proteins were in categories Q (12.8%), E, H (coenzyme
472 transport and metabolism), and W (extracellular structures), each at 10.3%.

473 ***Network and enrichment analyses***

Following COG analyses, we conducted STRING network and KEGG enrichment analyses (Table S2) to further characterize the metabolic alterations in fish sampled from each estuarine system. Consequently, the analysis of both up-regulated and down-regulated protein subsets allowed for a comprehensive evaluation of molecular metabolic changes within the liver. This approach allowed for the precise characterization of the dysregulation of specific metabolic pathways.

***Significant xenobiotic detoxification in flounder from all estuaries, less marked in the Aber Wrac'h***

The molecular signatures of xenobiotic biotransformation processes are commonly used as biomarkers in ecotoxicology. These processes that typically occur in the liver of an organism, are classified into two phases (Hassan et al. 2015). Phase I mechanisms involve mixed-function oxidases (MFOs), which result from the combined action of cytochrome P450 and NADPH-Cytochrome P450 reductase (Livingstone, 1998). MFOs activity serves as a recognized biomarker indicating the presence of contaminants such as PAHs, PCBs, and dioxins (Porte et al. 2000; Van der Oost et al. 2003). Phase II prepares for the excretion of xenobiotic intermediates formed during Phase I. This is achieved through the conjugation of metabolites to molecules such as glucuronic acid, amino acids, or glutathione by transferases. This process renders these metabolites highly hydrophilic and facilitates their elimination in the aqueous phase. The methionine cycle, associated with these detoxification mechanisms, also plays a crucial role. Methionine metabolism regulates two major homeostatic systems: cellular methylation and redox buffering. Methionine is an essential amino acid required for the synthesis of S-adenosylmethionine, the primary methylating agent in cells. It also serves as a precursor for amino acids like cysteine, which, in turn, contributes to the formation of glutathione, a key Phase II conjugating molecule and a cellular redox sensor. Previous studies have shown that the methionine cycle is often dysregulated in the livers of fish exposed to xenobiotics (Gandar et al. 2017; Galland et al. 2015). We searched for all proteins involved in xenobiotic detoxification processes (GO:0009410, Table 5). Concerning Phase I processes, Cytochrome P450 1A1 (CYP1A1) is clearly implicated in the hepatic detoxification of polycyclic aromatic hydrocarbons (PAHs) and other organic pollutants. This protein was significantly accumulated in fish from Douffine (2.29-fold), Aber Wrac'h (2.59-fold), and Guillec (2.65-fold). Another cytochrome P450, 2J2, showed increased accumulation in the livers of fish from Aber Wrac'h (1.57-fold). However, we identified no less than six cytochrome P450 proteins, five of which displayed reduced abundance in the livers of fish sampled from Quillimadec. These included cytochromes P450 2J2, 2B4, 2F2, and two isoforms of 2G1. Three of these were also diminished in Douffine, two in Flèche, and one in Guillec. Contrary to expectations, no cytochrome P450 proteins exhibited dysregulation in the livers of fish from Gouessant.

The accumulation of CYP1A1 suggested exposure to xenobiotics in Aber Wrac’h, Guillec, and Douffine. In contrast, the down-regulation of several other CYP proteins suggested lower exposure levels in fish from Quillimadec, Douffine, and Flèche compared to those from Aven. However, it is worth noting that certain pharmaceuticals have been shown to decrease CYP450 activity through reversible inhibition, non-receptor-

509 mediated mechanisms, or downregulation of receptor-mediated responses (Burkina et al. 2015). This has
510 been demonstrated in vitro for CYP1A (and CYP3A) by certain antifungals, such as clotrimazole (Burkina et al.
511 2013) and ketoconazole (Zlabek & Zamaratskaia, 2012).
512 Regarding Phase II proteins (Table 5), three proteins were up-regulated in fish livers from Gouessant, only
513 one is dysregulated in fish from other estuaries, and none in Aber Wrac'h individuals. The liver of Gouessant
514 flounders exhibited increased levels of Glutathione synthetase (1.96-fold), Glutathione peroxidase 1 (3.19-
515 fold), and Glutathione S-transferase theta-1 (2.28-fold). Glutathione synthetase catalyses the biosynthesis of
516 glutathione from gamma-glutamyl-cysteine and glycine. Glutathione plays a crucial role in detoxifying
517 xenobiotics and protecting cells against oxidative damage caused by free radicals. Glutathione peroxidase-1
518 is an intracellular antioxidant enzyme that reduces hydrogen peroxide to water. Glutathione S-transferase
519 theta 1 is involved in the detoxification of chemicals by conjugating reduced glutathione to various
520 electrophilic and hydrophobic compounds.
521 In estuaries along the French coast, high oyster GST activities were related to mixture of pesticides, the
522 metolachlor being the main molecule (Lerebours et al. 2023). Thus, the upregulation of phase II proteins in
523 Gouessant fish could suggest a detoxification linked to high level of pesticides in freshwater; this hypothesis
524 appears however unlikely, no convergent upregulation of the phase I being observed in this hydrosystem.
525 The Gouessant watercourse is fragmented by two successive dams, immediately upstream of the estuary,
526 which can influence the fate of pesticides and other contaminants. Because of their long hydraulic residence
527 time in large water volumes, particularly in summer, reservoirs and ponds can reduce considerably pesticide
528 concentration between upstream and downstream rivers, by favouring their accumulation and degradation
529 (Le Cor et al. 2021; Caquet et al. 2013; Devault et al. 2009). Thus, we suggest that the Gouessant dams
530 reduced downstream transfer of pesticides to the estuary, and that the flounder upregulation of phase II
531 proteins was possibly linked to the high production of hydrogen sulphide ($H_2S$) in the lower estuary and in
532 the Saint-Brieuc bay downstream, by bacteria metabolizing green macroalgae deposits (Pucheux et al. 2011;
533 Ménesguen et al. 2010); exposition of fish to $H_2S$ leading to lipid peroxidation and antioxidant responses (Liu
534 et al. 2022; Sreejai & Chithra, 2016).
535 A second hypothesis, explaining the dysregulation only for phase II could be the significant presence of
536 cyanobacteria detected in summer in Gouessant reservoirs, producing microcystins (most widely distributed
537 cyanotoxins), known to induce oxidative stress by promoting the formation of reactive oxygen species for
538 aquatic organisms (Welten et al. 2020; Du et al. 2019). Furthermore, microcystins upregulate glutathione S-
539 transferase and glutathione peroxidase in exposed fish (Le Manach et al. 2018). Cyanobacteria have been
540 detected in the Gouessant last dam reservoir (Pucheux et al. 2011), with concentrations of up to 204,000
541 cells.mL$^{-1}$ (danger threshold 100,000 cells.mL$^{-1}$). Cyanobacteria (oscillatoriales, microcystis and
542 synechococcus) have also been detected in the sediment downstream of the two dams, where flounder live
543 (Pucheux et al. 2011).

Alterations in the methionine cycle have often been associated with fish exposure to xenobiotics; methionine being a nutritionally essential sulphur-containing amino acid (Galland et al. 2015). In our inter-estuary comparison, we observed a highly significant enrichment of the KEGG category 'Cysteine and methionine metabolism' (KEGG pathway dre00270, Table S2) and specifically investigated the involved proteins (Table 5). We identified nine proteins from this cycle that were down-regulated in the livers of fish from Flèche. Six of these were down-regulated in fish from Douffine, and five in fish from Guillec. These proteins encompassed all stages of the methionine cycle and included two isoforms of betaine homocysteine methyl transferase (BHMT), three isoforms of S-adenosylmethionine (SAM) synthase, and four isoforms of adenosyl homocysteinase. This trend appears to mirror previous observations in other Brittany estuaries (Laurent et al. 2023). Similarly, the negative dysregulation of BHMT, SAM synthetase, and adenosylhomocysteinase suggested a preference for methionine synthesis over homocysteine. A vegetable washing unit present in the Flèche system directly discharges raw wastewater two kilometres upstream from the flounder collection site. Among the cleaned vegetables, there are many cruciferous vegetables (cauliflower, broccoli, romanesco,...) which naturally concentrate sulphur molecules: the glucosinolate and its metabolite, the phenethyl isothiocyanate (PEITC) (Castro-Torres et al. 2020, McNaughton & Marks, 2003); these molecules being toxic for bacteria, fungi and insects (Ali et al. 2018; Dufour et al. 2015). Furthermore embryos of *Salmo trutta* exposed to environmentally relevant concentration of PEITC (0.1 µg/L) showed increased mortality, teratogenic effects and impaired behaviour (White et al. 2019a). In the present study, we therefore hypothesize that washing several dozen tons of freshly harvested cruciferous vegetables per year could lead to the release of high levels of glucosinolate-PEITC into the river, that could explain the dysregulation of metabolism of sulphur compounds like methionine, in fish from Flèche.

Other proteins involved in xenobiotic detoxification mechanisms exhibited dysregulation as well. Most of these proteins showed dysregulation in fish from only one estuary. However, one enzymatic protein, 4-aminobutyrate aminotransferase (ABAT), appeared reduced in Guillec (FC=-2.37), Flèche (FC=-3.24), Quillimadec (FC=-2.12), Aber Wrac'h (FC=-3.51), and Douffine (FC=-3.62), but not in Gouessant. ABAT plays a role in the catabolism of gamma-aminobutyric acid (GABA), an important neurotransmitter and inhibitor of the central nervous system.

***Urea cycle slightly induced in flounder from the Gouessant, Quillimadec and Douffine estuaries***

The urea cycle (GO:0000050) has recently gained attention due to its potential link to exposure to nitrate and nitrite (Laurent et al. 2023). We investigated the urea cycle and found modifications in the livers of fish from the studied estuaries involving five proteins (Table 6): Argininosuccinate Lyase (ASL), Argininosuccinate Synthase (ASS), Carbamoyl phosphate synthetase II Aspartate transcarbamylase and Dihydroorotase (CAD), mitochondrial Aspartate Aminotransferase (ASAT), and cytoplasmic aspartate-tRNA ligase.

ASL, an enzyme catalysing the formation of arginine from Argininosuccinic Acid (ASA), was accumulated in Quillimadec (FC=1.57) and Douffine (FC=1.50). Arginine is subsequently decomposed into urea and ornithine,

579 which is excreted in urine to reduce cellular nitrogen compounds. Argininosuccinate synthase (ASS), involved
580 in the third step of the urea cycle, synthesizes argininosuccinate from citrulline and aspartate. ASS levels
581 were modified in Flèche (FC=-3.80), Quillimadec (FC=2.53), and Aber Wrac'h (FC=-2.38). CAD protein, while
582 not directly part of the urea cycle, can be activated in case of urea cycle dysregulation or saturation (Lee et
583 al. 2018). CAD was accumulated only in Douffine (FC=1.59).
584 Gouessant differed from other systems, with flounders displaying overexpression of mitochondrial aspartate
585 aminotransferase (FC=2.02) and cytoplasmic aspartate-tRNA ligase (FC=1.85) but not of typical urea cycle
586 enzymes. ASAT helps eliminating ammonia by producing aspartate, which enters the urea cycle, while
587 aspartate-tRNA ligase binds aspartate to its transfer RNA to form aspartyl-tRNA-Asp.
588 Surprisingly, no proteins associated with the urea cycle were dysregulated in fish livers from the Guillec
589 estuary, despite the presence of high loads of nitrogen in the water (as previously mentioned). The urea cycle
590 is responsible for converting excess ammonia into urea. This excess nitrogen typically results from the
591 breakdown of nitrogen-containing metabolites, such as proteins, amino acids, or ammonia itself, into urea,
592 a nitrogen compound that can be excreted (Mew et al. 2017). Our recent study (Laurent et al. 2023)
593 suggested a potential link between fish urea cycle dysregulation and exposure to a possible excess of
594 ammonium at the interface water-sediment, in sediments with a high oxygen deficit; this tend being detected
595 in the Horn highly eutrophicated estuary. Despite very similar high loads of nitrogen observed in the Guillec
596 water, this estuary shows mainly sandy bottoms whereas eutrophicated systems like the Horn display mostly
597 muddy sediments. Thus we suggest that the bacterial nitrate reduction activity in the Guillec estuarine
598 sediment is rather reduced and does not produce the excess of ammonium which is necessary for the
599 induction of urea cycle. While the connection between nitrate/nitrite exposure and the urea cycle (nitrogen
600 metabolism) remains unclear, previous studies showed urea cycle dysregulation following nitrite exposure in
601 Penaeus (Cheng & Chen, 2001).
602 Fish from Gouessant, Quillimadec, and Douffine accumulated proteins of the urea cycle. This finding can be
603 explained for the Gouessant by the relatively high levels of ammonium, nitrite, and nitrate, potentially
604 originating from large-scale pig farms, corn crops, and leading to an over production of green algae in the
605 bay downstream the estuary. In the Quillimadec estuary, intensive vegetable cultivation and contamination
606 from pig manure, likely contribute to urea cycle dysregulation in this estuary and also to green tide
607 production in the bay. The Douffine stream hosts three large fish farms close to the estuary that can lead to
608 important nitrogen loads. Indeed, fish farms can generate ammonium, nitrate and nitrite pollution (Helal et
609 al. 2017; Schenone et al. 2011), which may explain the molecular responses of flounder regarding the urea
610 cycle.

611 ***Flounder from Gouessant and Quillimadec estuaries showed responses to thermal stress***

612 Temperature is a critical water quality parameter in rivers and estuaries, influencing various ecological
613 processes. The proteomic analysis revealed alterations in several proteins associated with the response to

thermal stress (GO:0009408). This included the dysregulation of several Heat Shock Proteins (HSPs) and HSP-binding proteins (Table 7). The largest number of dysregulated proteins was observed in fish from the Gouessant estuary (6 proteins), followed by Quillimadec and Aber Wrac'h (4 proteins each).

HSPs are a highly conserved group of proteins with varying molecular weights (ranging from 16 to >100 kDa) found in all organisms, produced in response to cellular stress. In aquatic species, HSPs are known to be synthesized in response to changes in environmental temperature as well as other stress. Fish, in particular, express HSPs when exposed to thermal fluctuations, whether it involves an increase or decrease in temperature. As such, the expression of HSPs can be used as an indicator for environmental monitoring (Roberts et al. 2010).

The hepatic proteome of flounders from the Gouessant estuary exhibited an accumulation of proteins associated with the response to heat stress. Notable among these were two HSPs: 14-3-3 protein epsilon isoform X1 (FC=1.93) and HSP 60 kDa (FC=1.54), along with a homolog of Delta-1-pyrroline-5-carboxylate synthase (FC=2.43), which is involved in proline biosynthesis. Similarly, fish from Quillimadec displayed an accumulation of three HSPs: DNAJ homolog subfamily C member 3 (FC=2.38), HSP 70 kDa protein 4L (FC=1.51), and mitochondrial HSP 75 kDa (FC=1.57).

In contrast, the molecular signatures observed in fish from the other estuaries showed decreased levels of Stress-induced-phosphoprotein 1 in Guillec, Aber Wrac'h, and Douffine (FC=-1.88; -1.76; and -1.67, respectively), as well as a mitochondrial HSP 10 in Flèche and Aber Wrac'h (FC=-1.72 and -1.63). Fish from Flèche also exhibited a reduction in DNAJ homolog subfamily C member 3 (FC=-2.17).

During the sampling campaign, water temperature was measured in each estuary at the beginning of electric fishing, indicating an average temperature of 15.2 ± 2.2°C (Table S1); these point data do not allow to investigate the thermal regime of estuaries over the tidal cycle in summer. The Gouessant estuary showed the highest number of accumulated proteins associated with the thermal stress response. Indeed, Gouessant flows into a wide, shallow bay, which favours the heating up of water at rising tide during heat waves, coupled in effect with the two reservoirs above. As noted previously, the Gouessant watercourse showed two successive dams located immediately upstream the estuary which might expose the fish to warmer temperatures. Indeed, dams create reservoirs where water can warm up rapidly, particularly during summer. Furthermore, changes in temperature along the rivers are significantly influenced by the initial upstream temperature (Poole & Berman, 2001). As a result, water impoundment can disrupt water flow and alter the natural downstream hydrograph and thermograph (Daniels et al. 2020; Cai et al. 2018; Niemeyer et al. 2018). The Quillimadec estuary also showed significant responses to thermal stress. We suggest that the impoundment located immediately upstream the Quillimadec estuary could explain a thermal stratification in the reservoir producing warm waters in the estuary.

Another hypothesis is that fish from the Gouessant and Quillimadec estuaries may be exposed to other stressors, as HSPs are known to be induced by various stress conditions. Indeed, the trigger for the synthesis of these chaperone proteins is the presence of denatured proteins within cells, which can result from multiple

650 harmful environmental conditions. Finally, it is worth noting that HSPs also play a role in the immune system,
651 inflammatory response, and apoptosis (Roberts et al. 2010), which may be related to the high number of
652 dysregulated proteins associated with the immune system observed in our study (see later).

653 ***Flounder from Gouessant estuary showed molecular signatures of hypoxia***

654 Physico-chemical parameters measured in the estuaries on the day of fish sampling indicated oxygen
655 concentrations ranging from 8.74 to 10.85 mg $O_2$.$L^{-1}$, well above the hypoxia threshold of 2 mg $O_2$.$L^{-1}$ (Vaquer-
656 Sunyer & Duarte, 2008) generally accepted as critical for benthic marine organisms. Nevertheless, previous
657 proteomic analyses have uncovered a response to thermal stress in flounders from certain systems, which
658 may elucidate the observed dysregulations of molecular markers linked to the response to hypoxia
659 (GO:0001666). A total of eleven proteins associated with the response to hypoxia exhibited dysregulation in
660 our study (Table 8).
661 Hypoxia upregulated protein 1 (HYOU1) and Glycogen phosphorylase muscle form (PYGM) displayed the
662 most significant dysregulation. HYOU1 showed decreased abundances in fish captured from the Guillec (FC=-
663 1.62) and Flèche (FC=-2.29) estuaries but was accumulated in Quillimadec (FC=1.73). An isoform of PYGM
664 was accumulated in the livers of individuals from Le Guillec (FC=1.54), Flèche (FC=1.87), and Quillimadec
665 (FC=1.57), and another isoform in Flèche (FC=1.83).
666 HYOU1 plays a cytoprotective role in mitochondrial repair by suppressing hypoxia-induced premature cell
667 death. HYOU1 is part of the Heat Shock Proteins (HSPs) family and assists in protein folding and secretion in
668 the endoplasmic reticulum (ER). Under hypoxic conditions, HYOU1 accumulates in the ER (Chene et al. 2006).
669 PYGM serves as a key enzyme in the initial stage of glycogenolysis, supplying energy for muscle contraction.
670 Elevated expression levels of PYGM (along with Hif1-α) have been observed in *Larimichthys crocea* under
671 hypoxic conditions to ensure rapid energy delivery during hypoxic stress (Ding et al. 2022). In contrast, Delta-
672 aminolevulinic acid dehydratase, an enzyme involved in the early stages of heme biosynthesis (critical for
673 oxygen transport in organisms), showed a decreased abundance specifically in Quillimadec (FC=-2.83).
674 Gouessant flounders showed distinct protein dysregulations compared to those observed for the other
675 systems. Indeed, they exhibited over-activation of Alpha-1-antitrypsin (A1AT) homolog (FC=2.04), Prohibitin-
676 2 (Phb-2) isoform X1 (FC=1.81), and of NDRG1 (FC=3.41). Conversely, L-lactate dehydrogenase A (LDHA) and
677 Protein-tyrosine kinase 2-beta (PTK2B) were under expressed (FC=-2.11 and -3.00, respectively). A1AT is a
678 serine protease inhibitor (serpin) that protects tissues against enzymes produced by inflammatory cells.
679 PHBs, highly conserved proteins, are found in various cellular compartments (Mishra et al. 2006) and have
680 been identified in the inner mitochondrial membrane. Mitochondrial prohibitin complexes control cell
681 proliferation, cristae morphogenesis, and the functional integrity of mitochondria (Merkwirth & Langer,
682 2009). The NDRG1 protein has recently been assigned a distinct role as a molecular switch for hypoxia
683 adaptation in Danio rerio (Park et al. 2022). Finally, LDH catalyses the conversion of pyruvate to lactate in
684 fermentative processes that are favoured under conditions of oxygen limitation (oxygen being the final

electron acceptor in respiration). Additionally, the regulatory region of the LDH gene contains essential binding sites for HIF1, which govern its regulation in response to hypoxia. In the black rockfish (*Sebastes schlegelii*), the LDHA signalling pathway is activated by HIF1 to promote anaerobic glycolysis essential for coping with the increased energy demands induced by hypoxic stress (Qin et al. 2023; Sun et al. 2020; Gong et al. 2020). Considering that we previously reported the highest Mn:Ca ratio in the otoliths of Gouessant fish, which suggested chronic exposure to hypoxia, the dysregulated molecular markers in these fish are likely linked to this chronic exposure. The proliferation of green algae highlighted above in the bay downstream the Gouessant estuary, can also be at the origin of or contribute to the hypoxic phenomena observed, particularly at rising tide.

Despite the results above, proteomic analyses failed to detect proteins more directly associated with the response to hypoxia, such as Hypoxia Inducible Factor 1 alpha (HIF1a), Vascular Endothelial Growth Factor (VEGF), angiopoietin, or haemoglobin. In a previous study dealing with other small estuaries from Brittany (Laurent et al. 2023), these molecular markers were markedly dysregulated in several estuaries exposed to hypoxic events. However, this previous study used transcriptomic methods to identify the mentioned hypoxia response genes. Hence, either the hypoxic episodes were less severe than those observed in Laurent et al. (2023), or proteomics may be less effective than transcriptomics in revealing components of the molecular responses of flounder to hypoxic events.

***Stimulation of fatty acids oxidation***

We have observed that hypoxia affects fish's ability to produce energy, but the primary energy reserve in fish is their lipid stores. Another category of proteins that was clearly dysregulated relates to fatty acid metabolism (GO:0006631) (Table 9). We notably observe a significant decrease in acetyl-CoA carboxylase (FC=-4.05 and -4.50 in Quillimadec and Aber Wrac'h, respectively), responsible for malonate synthesis, which is the first step in fatty acid synthesis, and fatty acid synthase itself is reduced in the livers of fish from Guillec, Quillimadec, and Aber Wrac'h.

While this modification did not appear so clearly in fish sampled from the other estuaries, we can clearly see some signs of enhanced lipid degradation in general (evidenced, for example, by a 1.88-fold induction of bile salt-activated lipase in Gouessant) and specifically in fatty acids. Indeed, we find numerous proteins involved in the beta-oxidation of fatty acids (GO:0019395) accumulated in the livers of fish from all the estuaries studied. This included several isoforms of acyl-CoA synthetases, acyl-CoA and hydroxyacyl-CoA dehydrogenases, beta-ketothiolase, as well as 2,4 dienoyl-CoA reductase involved in the degradation of unsaturated fatty acids.

The accumulation profiles of these proteins suggest that this metabolism is highly stimulated in Douffine and Flèche (5 proteins from the GO:0019395 category accumulated, with FC ranging from 1.52 to 3), but also in Aber Wrac'h (4 proteins), Guillec, and Gouessant (3 proteins). Only one protein from this category is accumulated in fish from Quillimadec. Fatty acid beta-oxidation constitutes the most efficient energy

720 metabolism in animals, and its stimulation in the livers of fish from nearly all estuaries likely responds to an
721 increased energy demand.

722 ***Immune system, the first response mechanism to stress, especially for flounder from Gouessant***

723 Proteomic analyses have revealed significant dysregulation of a large number of proteins associated with the
724 immune system (Table 10), especially those related to the complement system (GO:0006955 and
725 GO:0006956). Complement proteins correspond to the set of enzymes involved in the organism's immune
726 defence. The complement system serves as a bridge between innate and acquired immunity, facilitating
727 enhanced antibody responses, immunological memory, lysis of foreign cells, and the clearance of antigen-
728 antibody complexes and apoptotic cells (Lubbers et al. 2017).
729 Fish sampled in the Guillec estuary exhibited the fewest dysregulated proteins related to complement, with
730 2 up-regulated and 4 down-regulated proteins. Flounder captured in Quillimadec and Douffine displayed 11
731 dysregulated proteins, while those from Flèche and Aber Wrac'h showed 12 dysregulated proteins. In
732 contrast, fish from the Gouessant estuary presented the highest number of dysregulated proteins associated
733 with the complement system, featuring 12 up-regulated and 3 down-regulated proteins (Table 10).
734 KEGG enrichment analysis revealed the overexpression of proteins linked to "Salmonella infection" (KEGG
735 pathway dre05132, Table S2) exclusively in flounder from the Gouessant estuary. The dysregulation of this
736 pathway reflects the development of an immune response specific to fish from Gouessant. The general
737 dysregulation of the immune system in fish from Gouessant could also be linked to the presence of $H_2S$ in
738 the estuary and in the Saint Brieuc bay downstream (Pucheux et al. 2011; Ménesguen et al. 2010). Several
739 studies have already demonstrated dysregulation of genes associated with the immune response (Lazado et
740 al. 2024; Leeuwis & Gamperl, 2022), following exposure to $H_2S$.
741 Among the numerous dysregulated proteins, Complement C3 and Complement factor B both exhibited
742 decreased levels in the livers of flounder from Guillec (FC=-1.81; -1.56), Aber Wrac'h (FC=-2.00; -1.92), and
743 Douffine (FC=-1.61; -1.56). These two proteins were not significantly dysregulated in fish from Gouessant,
744 Flèche, and Quillimadec. While Complement factor 3 is one of the most abundant proteins in serum and plays
745 a pivotal role in complement activation, Complement factor B is a component of the alternative complement
746 pathway, contributing to complement activation in response to bacterial invasion (Laskowski & Thurman,
747 2018; Sunyer et al. 1997; Li & Sun, 2017). The absence of detection of these two complement proteins in fish
748 from Guillec, Aber Wrac'h, and Douffine, compared to those from the reference estuary (Aven), suggests a
749 potentially weaker immune response in these three systems.
750 Likewise, three distinct Major Vault like Proteins (MVPs) exhibited decreased levels exclusively in the hepatic
751 proteome of flounder from Aber Wrac'h. Chronic exposure of epidermal cells to low levels of benzo-a-pyrene
752 (BAP) has been linked to MVP overexpression in previous studies (Cheng et al. 2000). Although MVPs are
753 abundant in various cell types, including macrophages, dendritic cells, epithelial cells, and liver neoplasia,
754 their precise functions remain elusive. Juvenile mullets (*Chelon labrosus*) exposed to heavy fuel oil displayed

significantly up-regulated hepatic MVPs (De Cerio et al. 2012). Consequently, the under-detection of MVPs in flounder from Aber Wrac'h, compared to those from Aven, could be associated with chronic PAH exposure across all systems, including Aven.

Finally, the analysis of the liver proteome revealed the dysregulation of several proteins associated with the inflammatory response, such as Cathepsin B-like, Leukotriene A-4 hydrolase, and Plasma alpha-L-fucosidase, and proteins linked to lymphocyte activation, such as Dipeptidyl peptidase 1 and the biased Plastin-2.

### 3.5. Updating the typologies of the studied estuaries in the light of proteomics

The interpretation of the preliminary principal component analysis based on environmental metrics (geography, hydrobiology, pollutant chemistry and targeted flounder environmental signatures: Fig. 3), was combined with the proteomic data, to establish a precise typology of the seven small estuaries in Brittany.

The **Aven** watershed still appeared to be the system least affected by human activities. Indeed, the flounders sampled in this estuary showed the lowest levels of metals in their muscle, with the exception of relatively high values of arsenic. The source of arsenic contamination in Aven is not clearly identified at this time. However, no induction of arsenate methyltransferase was observed in Aven flounders, in contrast to Laurent et al. (2023). In this estuary, a small harbour and a car park could explain a moderate contamination by PAHs. In addition, low values of nitrite and nitrate loads were detected in freshwater. Indeed, agricultural pressure is quite moderate, and mainly oriented towards the production of canned vegetables, leading to a low concentration of water pesticides. At last, the Aven basin showed a high density of bocage which plays a major role in the purification of runoff water; hedges and embankments increasing the runoff time of rainwater and thus facilitating its infiltration into the soil (Caubel-Forget et al. 2001). The diachronic analysis of the Aven watershed is a relevant and stable reference hydrosystem for flounder -omics approaches.

On the other hand, the **Gouessant** watershed emerged as the most anthropised and stressed among the studied hydrosystems. Thus, the Gouessant is characterized by heavy agricultural pressure, high levels of pesticides, nitrite and nitrate in freshwater related to intensive cereal cultivation and pig farming; the hedge density being particularly reduced in this watershed. Furthermore, we suggest that a low pesticide concentration is very likely in the Gouessant estuarine waters, due to the presence of two dams reducing downstream transfer of pesticides and favouring their degradation. Thus, the activation of phase II xenobiotic detoxification could be a fish response to oxidative stress related to (1) $H_2S$ produced by bacteria metabolizing green macroalgae deposits in the estuary, and (2) a chronic exposure to cyanotoxins generated in the eutrophicated reservoirs and transferred to the estuary. The fish antioxidant activity may help to restore the liver function after a cyanotoxins contamination (Paulino et al. 2020; Falfushynska et al. 2023). Furthermore, flounders from the Gouessant showed an induction of the urea cycle related to excess of nitrogen, hypoxia and heat stress. The marked dysregulation of proteins related to immune system in the Gouessant could be the signal of severe immune disorders induced by cyanotoxins in rivers (Falfushynska et al. 2023).

The four adjacent watersheds, Guillec, Flèche, Quillimadec and Aber Wrac'h, were apparently similar, with high agricultural pressure linked to vegetable and cereal crops, animal production and high inputs of nitrogen. However, we have been able to differentiate them, thanks to proteomics.

The **Guillec** watershed was mainly characterized by intensive production of vegetables and the highest levels of pesticide treatment frequency and freshwater pesticide concentration over the whole hydrosystems. Fish responses to stressors in the Guillec were mainly characterized by xenobiotic detoxification with a dysregulation of Cytochrome P450 1A1 and other proteins linked to the methionine cycle; this detoxification being probably related to the massive use of pesticides over the whole watershed.

The agricultural activities in the **Flèche** basin were based on vegetable production, but displayed also a high level of livestock. The cocktail of water pesticides in Flèche and Guillec appeared rather similar, the level of pesticide being reduced by a third in Flèche. The major molecular response of the Flèche fish was a highly marked dysregulation of the methionine cycle, no apparent dysregulation being observed in phase I and phase II detoxification. Thus we suggest that this alteration in the methionine cycle could be associated with fish exposure to pesticides, but possibly also to the wastewaters of a large vegetable washing unit producing a high flux of glucosynolate - phenetyl isothyocyanate in the downstream river. Furthermore, Flèche fish showed a stimulation of fatty acids oxidation, an efficient way to produce energy to respond to the significant increase of the metabolic rate particularly detected in this stressed hydrosystem.

The level of water pesticide appeared relatively limited in the **Quillimadec** watershed *vs* the previous systems (Gouessant, Guillec, Flèche). Thus we observed in this watershed a moderate fish xenobiotic detoxification, but an induction of the urea cycle linked to a regular input of nitrogen in the estuary, related to agricultural activities and relatively high human population density. The fish response to thermal stress was probably linked to warm waters coming from the reservoir upstream the estuary.

The **Aber Wrac'h** hydrosystem appeared as the least impacted by stressors considering the four agricultural adjacent watersheds (Guillec, Flèche, Quillimadec, Aber Wrac’h), except for an arsenic contamination. Thus, proteomics revealed a moderate fish xenobiotic detoxification activity.

The **Douffine** stood out from the other hydrosystems due to multi-metallic contamination related to past mining operations, and very high fish farm production. The agricultural activities were moderate over this watershed which displayed the lowest level of water pesticides. Possible effluents of industrial activity and domestic wastes of a small town just upstream the estuary could explain the dysregulation of Phase I xenobiotics detoxification. The intensive fish farming activity in the Douffine induced high nitrogen loads and hence eutrophication, possibly explaining the dysregulation of the urea cycle observed with proteomics. A general hypoxia was clearly detected in the estuary considering the high level of manganese otolith concentration. Thus, the absence of molecular fish response to hypoxia in the Douffine was probably related to normoxia over a period of two to three days before the fish sampling. Finally, Douffine fish showed a dysregulation of proteins involved in the degradation of unsaturated fatty acids metabolism, probably due to the wastes of fish farming impacting lipid metabolism of wild fish (White et al. 2019b).

## 4. Conclusion

In the general context of stress ecology, we explored in the present paper the health status of small estuarine systems included in agricultural watersheds in Brittany - France. Our approach combined data on watershed geography, water and sediment chemistry, and biology of the estuarine flatfish *P. flesus*, including environmental signatures and shotgun proteomics. Data at different spatial and temporal scales were collected and analysed, in order to establish a high quality diagnostic of estuaries. The largest scale was obtained with geography and land use, which gives an overall view of the watersheds landscape. The finest scale was obtained with molecular approaches and more particularly proteomics performed at fish individual scales to get insights of the fish liver whole metabolism. The proteome is mainly affected by short/medium term changes in estuarine water conditions.

The different approaches presented in the present article were complementary and allowed to define an accurate typology of anthropization in the different watersheds. Regarding our main hypothesis, proteomics analyses have significantly improved the qualification and quantification of ecological quality of estuaries, disentangling the functioning and the respective status of the seven hydrosystems. For instance, in the four adjacent agricultural hydrosystems, fish liver metabolisms differed. Flounders from Guillec, Flèche and Quillimadec showed differential signatures of exposure to stressors (hypoxia, pesticides, excess of nitrogen, thermal stress). We suggest that the better ecological status of the Aber Wrac'h hydrosystem can be explained by better conservation of its, higher natural surfaces and riparian vegetation over the watershed. Our multidisciplinary approach considering a juvenile fish model that have spent five consecutive summer months in estuary offers a holistic perspective, departing from traditional point measurements to assess water quality, thus enabling a more accurate assessment of the aquatic environment and threats to local biodiversity. Thus, the methodology proposed in the present paper could produce relevant tools for assessing the ecological quality of estuaries and developing new management and restoration strategies for anthropised watersheds.

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**Table 1.** Geographical metrics from Gouessant, Guillec, Flèche, Quillimadec, Aber Wrac'h, Douffine and Aven hydrosystems.

| Geographical Metrics | GOUESSANT | GUILLEC | FLÈCHE | QUILLIMADEC | ABER WRAC'H | DOUFFINE | AVEN |
|---|---|---|---|---|---|---|---|
| Catchment area ($km^2$) | 420.2 | 72.5 | 73.5 | 79.2 | 95.6 | 173.9 | 193.7 |
| Total Livestock ($UGBTA.ha^{-1}$)* | 284.7 | 328.9 | 533.5 | 361.4 | 362.2 | 221.7 | 187.9 |
| Agricultural surfaces (% of catchment) | 74.9 | 74.4 | 77.7 | 72.2 | 71.4 | 59.1 | 70.3 |
| Pesticides treatments ($IFT.catchment^{-1}$) | 2.7 | 3.8 | 2.5 | 2.5 | 2.4 | 1.8 | 2.1 |
| Fish farm ($T.year^{-1}$) | 0 | 450 | 20 | 50 | 0 | 900 | 430 |
| Population density ($inhab.km^{-2}$) | 84.3 | 85.7 | 45.9 | 172.4 | 117.8 | 31.3 | 77.8 |
| Urbanised surfaces (% of catchment) | 10.9 | 18.2 | 12.9 | 19.1 | 18.7 | 5.6 | 10.9 |
| Natural surfaces (% of catchment) | 14.2 | 7.5 | 9.5 | 8.7 | 9.9 | 35.3 | 18.8 |
| Hedges density ($m.km^{-2}$) | 5628 | 7546 | 9554 | 9555 | 7724 | 7970 | 9839 |
| Riparian vegetation (% 100 m river band) | 16.6 | 17.6 | 16.9 | 12.9 | 18.3 | 33.3 | 25.4 |

** UGBTA correspond to the total feed intake of the livestock, i.e. the number of animals based on their complete feed intake*

**Table 2.** Mean and standard error of chemical and biological analysis realised on samples from Gouessant, Guillec, Flèche, Quillimadec, Aber Wrac'h, Douffine and Aven hydrosystems, in 2020. Nitrites, nitrates, and ammonium concentrations in water samples. Organic pollutants concentrations in *P. flesus* tissue. Trace elements concentrations in *P. flesus* muscle. Trace elements concentrations in sediments. Trace elements concentrations in *P. flesus* otoliths. (Statistics: Kruskal-Wallis test: p-value < 0.05, letters correspond to significant differences among estuaries).

| Chemical analysis | | GOUESSANT | GUILLEC | FLÈCHE | QUILLIMADEC | ABER WRAC'H | DOUFFINE | AVEN |
|---|---|---|---|---|---|---|---|---|
| **Nitrogen** | $NO_2^-$ | $0.16 \pm 0.03^{(a)}$ (0.11)** | $0.14 \pm 0.03^{(a)}$ (0.17)** | $0.06 \pm 0.01^{(abc)}$ (0.00029)** | $0.07 \pm 0.03^{(abc)}$ (0.14)** | $0.05 \pm 0.01^{(bc)}$ (0.06)** | $0.12 \pm 0.04^{(ab)}$ (0.08)** | $0.03 \pm 0.002^{(c)}$ (0.016)** |
| **concentrations ($mg.L^{-1}$)** | $NO_3^-$ | $21.1 \pm 3.6^{(ad)}$ (25.2)** | $50.8 \pm 1.5^{(b)}$ (48.0)** | $36.8 \pm 2.7^{(c)}$ (51.2)** | $33.7 \pm 2.7^{(ac)}$ (32.1)** | $32.8 \pm 1.8^{(acd)}$ (19.2)** | $12.0 \pm 0.9^{(d)}$ (18.5)** | $24.0 \pm 1.5^{(acd)}$ (25.0)** |
| **in water samples*** | $NH_4^+$ | $0.15 \pm 0.03^{(ab)}$ | $0.4 \pm 0.09^{(a)}$ | $0.06 \pm 0.01^{(bcd)}$ | $0.09 \pm 0.02^{(abc)}$ | $0.07 \pm 0.02^{(cd)}$ | $0.1 \pm 0.03^{(abc)}$ | $0.03 \pm 0.01^{(d)}$ |
| **Organic pollutants concentrations ($ng.g^{-1}$ DW) in sediment** | PAHs | $117 \pm 5^{(a)}$ | $105 \pm 68^{(a)}$ | $300 \pm 103^{(ab)}$ | $488 \pm 40^{(ab)}$ | $274 \pm 43^{(ab)}$ | $536 \pm 162^{(ab)}$ | $1480 \pm 270^{(b)}$ |
| | PCBs | <DL | <DL | <DL | <DL | <DL | <DL | <DL |
| **Organic pollutants concentrations ($ng.g^{-1}$ DW) in fish tissue** | PCBs | $4.0 \pm 1.2^{(a)}$ | $0.7 \pm 0.7^{(a)}$ | $11.6 \pm 0.5^{(abc)}$ | $22.8 \pm 4.4^{(bc)}$ | $4.3 \pm 1.2^{(ab)}$ | $27.7 \pm 0.2^{(c)}$ | $22.1 \pm 4.6^{(c)}$ |
| **Trace elements concentrations ($\mu g.g^{-1}$ DW) in sediment** | Arsenic | $4.3 \pm 0.4^{(a)}$ | $8.4 \pm 0.2^{(ac)}$ | $4.1 \pm 0.7^{(a)}$ | $9.2 \pm 0.7^{(ab)}$ | $27.4 \pm 3.4^{(b)}$ | $14.3 \pm 0.4^{(bc)}$ | $13.1 \pm 0.7^{(bc)}$ |
| | Cadmium | $0.11 \pm 0.01^{(a)}$ | $0.58 \pm 0.01^{(bc)}$ | $0.15 \pm 0.02^{(ab)}$ | $0.32 \pm 0.03^{(abd)}$ | $0.29 \pm 0.06^{(abd)}$ | $2.18 \pm 0.3^{(c)}$ | $0.87 \pm 0.03^{(cd)}$ |
| | Copper | $5.9 \pm 0.6^{(ab)}$ | $7.0 \pm 0.1^{(abd)}$ | $2.7 \pm 0.5^{(a)}$ | $7.4 \pm 0.5^{(abd)}$ | $13.8 \pm 2.1^{(bc)}$ | $69.2 \pm 13.3^{(c)}$ | $25.4 \pm 1.3^{(cd)}$ |
| | Lead | $9.8 \pm 0.9^{(ad)}$ | $11.6 \pm 0.3^{(ab)}$ | $5.5 \pm 0.5^{(a)}$ | $11.5 \pm 0.6^{(ab)}$ | $34.7 \pm 5.6^{(bc)}$ | $261.7 \pm 56.9^{(c)}$ | $31.6 \pm 0.9^{(bcd)}$ |
| | Zinc | $23.5 \pm 2.3^{(ab)}$ | $69.6 \pm 1.5^{(ac)}$ | $16.2 \pm 2.7^{(b)}$ | $47.7 \pm 3.3^{(abd)}$ | $60.2 \pm 8.6^{(abc)}$ | $326.1 \pm 51.5^{(c)}$ | $110.9 \pm 5.7^{(cd)}$ |
| **Trace elements concentrations ($\mu g.g^{-1}$ DW) in fish muscle** | Arsenic | $1.72 \pm 0.38^{(ac)}$ | $2.16 \pm 0.24^{(a)}$ | $0.39 \pm 0.01^{(b)}$ | $2.83 \pm 0.33^{(a)}$ | $3.12 \pm 0.51^{(a)}$ | $0.8 \pm 0.09^{(bc)}$ | $6.02 \pm 0.32^{(d)}$ |
| | Cadmium | $0.001 \pm 0.0002^{(ace)}$ | $0.003 \pm 0.0002^{(bd)}$ | $0.004 \pm 0.0004^{(be)}$ | $0.001 \pm 0.0001^{(a)}$ | $0.002 \pm 0.0003^{(cd)}$ | $0.003 \pm 0.001^{(bd)}$ | $0.001 \pm 0.0002^{(e)}$ |
| | Copper | $1.06 \pm 0.08^{(a)}$ | $1.07 \pm 0.04^{(a)}$ | $0.93 \pm 0.15^{(b)}$ | $0.96 \pm 0.05^{(ab)}$ | $0.96 \pm 0.07^{(ab)}$ | $1.03 \pm 0.05^{(a)}$ | $0.81 \pm 0.04^{(b)}$ |
| | Lead | $0.03 \pm 0.02^{(ab)}$ | $0.02 \pm 0.005^{(ab)}$ | $0.01 \pm 0.01^{(a)}$ | $0.02 \pm 0.003^{(bc)}$ | $0.02 \pm 0.003^{(bcd)}$ | $0.05 \pm 0.01X^{(c)}$ | $0.01 \pm 0.001^{(ad)}$ |
| | Zinc | $44.5 \pm 9.8^{(a)}$ | $33.6 \pm 3.1^{(a)}$ | $27.9 \pm 1.0^{(a)}$ | $28.4 \pm 3.2^{(ab)}$ | $34.3 \pm 4.2^{(a)}$ | $32.3 \pm 2.9^{(a)}$ | $21.9 \pm 1.5^{(b)}$ |
| **Trace elements concentrations ($\mu mol.mol^{-1}$ Ca) in fish otoliths** | Strontium | $423 \pm 99^{(a)}$ | $471 \pm 84^{(a)}$ | $650 \pm 45^{(ab)}$ | $811 \pm 93^{(bc)}$ | $1155 \pm 36^{(cd)}$ | $478 \pm 27^{(a)}$ | $1655 \pm 39^{(d)}$ |
| | Manganese | $36.5 \pm 10.5^{(ac)}$ | $4.9 \pm 1.2^{(bd)}$ | $7.0 \pm 0.9^{(b)}$ | $5.4 \pm 0.8^{(b)}$ | $9.9 \pm 2.1^{(ab)}$ | $30.6 \pm 3.9^{(c)}$ | $1.8 \pm 0.2^{(d)}$ |
| | Copper | $0.46 \pm 0.22^{(a)}$ | $0.26 \pm 0.08^{(abc)}$ | $0.15 \pm 0.03^{(bc)}$ | $0.48 \pm 0.25^{(ab)}$ | $0.26 \pm 0.03^{(a)}$ | $0.23 \pm 0.06^{(abc)}$ | $0.16 \pm 0.03^{(c)}$ |
| | Zinc | $11.6 \pm 6.6^{(a)}$ | $3.3 \pm 0.8^{(a)}$ | $2.3 \pm 0.5^{(a)}$ | $1.2 \pm 0.4^{(b)}$ | $4.4 \pm 2.2^{(ab)}$ | $9.1 \pm 7.9^{(ab)}$ | $3.8 \pm 2.1^{(ab)}$ |

** Data provided by the Regional Directorate for the Environment, Development and Housing (DREAL) of Rennes for 2019 water samples, excepted for Aven (2016).*

*** Additional values in brackets correspond to nitrites and nitrates concentrations measured the sampling day in 2020.*

**Table 3.** Mean concentrations of pesticides used in agriculture and detection frequencies of major molecules in freshwater in Gouessant, Guillec, Flèche, Quillimadec, Aber Wrac'h, Douffine and Aven hydrosystems, in 2018-2019 (after rainy events).

| Estuaries | GOUESSANT | GUILLEC | FLÈCHE | QUILLIMADEC | ABER WRAC'H | DOUFFINE | AVEN |
|---|---|---|---|---|---|---|---|
| **Total cumulative pesticide concentrations in water (µg.L$^{-1}$)** | **6.49** | **6.61** | **2.12** | **1.25** | **1.85** | **1.16** | **1.45** |
| Major molecules detected (detection frequencies %) | AMPA (95%)<br>Chlorpropham (90%)<br>Diflufenicanil (50%)<br>Glyhosate (95%)<br>Metazachlor ESA (82%)<br>Metolachlor (70%)<br>Metolachlor ESA (100%)<br>Propiconazole (70%)<br>Tebuconazole (65%) | 2,6 dichloro-benzamide (100%)<br>Acetochlor ESA (100%)<br>Alachlor ESA (100%)<br>AMPA (72%)<br>ASDM (84%)<br>Atrazine desethyl (84%)<br>Boscalid (61%)<br>Dimetomorph (68%)<br>Metazachlor ESA (100%)<br>Metazachlor OXA (100%)<br>Metolachlor ESA (100%)<br>Metolachor OXA (50%)<br>Oxadixyl (100%) | 2,6 dichloro-benzamide (100%)<br>Acetochlor ESA (100%)<br>AMPA (50%)<br>ASDM (90%)<br>Atrazine desethyl (90%)<br>Glyphosate (100%)<br>Metazachlor ESA (100%)<br>Metolachlor ESA (100%)<br>Metolachlor OXA (75%)<br>Trichlopyr (50%) | 2,6 dichloro-benzamide (50%)<br>Acetochlor ESA (100%)<br>Alachlor ESA (50%)<br>AMPA (50%)<br>ASDM (100%)<br>Atrazine desethyl (90%)<br>Metazachlor ESA (100%)<br>Metolachlor ESA (100%)<br>Metolachor OXA (50%) | 2,6-Dichlorobenzamide (87%)<br>AMPA (69%)<br>Atrazine desethyl (80%)<br>Glyphosate (51%)<br>Metazachlor ESA (100%)<br>Metolachlor ESA (100%) | Metazachlor ESA (66%)<br>Metazachlor OXA (44%)<br>Metolachlor ESA (88%) | Acetochlor ESA (77%)<br>Alachlor ESA (77%)<br>ASDM (100%)<br>Atrazine desethyl (55%)<br>Metazachlor ESA (77%)<br>Metolachlor ESA (77%) |
| Data availability (wartershed managers) | Service Bassins versants et GEMAPI<br>Direction Environnement Lamballe Terre & Mer | Syndicat Mixte de Production et de Transport d'Eau de l'Horn | Syndicat Mixte des Eaux du Bas-Léon | Syndicat Mixte des Eaux du Bas-Léon | Syndicat Mixte des Eaux du Bas-Léon | Etablissement Public d'Aménagement et de Gestion du bassin versant de l'Aulne | Cornouaille Agglomération Centre technique<br>SAGE Sud Cornouaille |

**Table 4.** Number of dysregulated proteins in *P. flesus* liver in Gouessant, Guillec, Flèche, Quillimadec, Aber Wrac'h and Douffine *vs* Aven.

| Proteomics | GOUESSANT | GUILLEC | FLÈCHE | QUILLIMADEC | ABER WRAC'H | DOUFFINE |
|---|---|---|---|---|---|---|
| Up-regulated | 202 | 44 | 58 | 110 | 33 | 71 |
| Down-regulated | 46 | 45 | 75 | 61 | 60 | 39 |

**Table 5.** List of dysregulated proteins related to xenobiotics detoxification in *P. flesus* liver, in Gouessant, Guillec, Flèche, Quillimadec, Aber Wrac'h and Douffine *vs* Aven.

| | Accession | Name | GO Term | Gouessant | Guillec | Flèche | Quillimadec | Aber Wrac'h | Douffine |
|---|---|---|---|---|---|---|---|---|---|
| **Phase I xenobiotics detoxification processes GO:0009410** | XP_019935791.1 | Cytochrome P450 1A1 | - | - | 2.65 | - | - | 2.59 | 2.29 |
| | XP_019936473.1 | Cytochrome P450 2J2-like | x | - | - | - | -2.71 | 1.57 | -2.59 |
| | XP_019957767.1 | Cytochrome P450 2B4-like isoform X1 | - | - | - | -1.79 | -1.79 | - | -2.5 |
| | XP_019957775.1 | Cytochrome P450 2F2-like | - | - | -2.32 | -4.5 | -2.67 | - | -3.43 |
| | XP_019943606.1 | Cytochrome P450 2G1-like | - | - | - | - | -1.96 | - | - |
| | XP_019943823.1 | Cytochrome P450 2G1-like | - | - | - | - | -2 | - | - |
| | XP_019936740.1 | Dimethylaniline monooxygenase [N-oxide-forming] 5-like | x | - | | 1.86 | - | - | - |
| **Phase II xenobiotics detoxification processes GO:0009410** | XP_019945053.1 | Glutathione synthetase | x | 1.96 | - | - | - | - | - |
| | XP_019942157.1 | Glutathione peroxidase 1-like | x | 3.19 | - | - | - | - | - |
| | XP_019945879.1 | Glutathione S-transferase A-like | - | - | 1.51 | 1.53 | - | - | - |
| | XP_019958382.1 | Glutathione S-transferase theta-1-like | - | 2.28 | - | - | - | - | - |
| | XP_019969103.1 | UDP-glucuronosyltransferase-like | x | - | - | - | 2.22 | - | - |
| | XP_019969171.1 | UDP-glucuronosyltransferase 2A1-like partial | x | - | - | - | - | - | -1.50 |
| **Methionine cycle** | XP_019967225.1 | Adenosylhomocysteinase | - | -1.54 | -1.55 | -1.82 | - | - | -1.8 |
| | XP_019942595.1 | Adenosylhomocysteinase 2-like | - | - | - | -1.63 | - | - | - |
| | XP_019935095.1 | Adenosylhomocysteinase 2-like isoform X1 | - | 3.44 | - | -1.51 | - | - | - |
| | XP_019954766.1 | Betaine-homocysteine S-methyltransferase 1-like | - | - | -2.15 | -4.08 | - | -2.40 | -3.60 |
| | XP_019954768.1 | Betaine-homocysteine S-methyltransferase 1 | - | - | - | -2.27 | - | - | -1.76 |
| | XP_019948036.1 | Putative adenosylhomocysteinase 3 | - | - | - | -1.61 | - | - | - |
| | XP_019953264.1 | S-adenosylmethionine synthase-like | - | - | -1.53 | -1.71 | - | - | -2.11 |
| | XP_019965459.1 | S-adenosylmethionine synthase | - | - | -1.60 | -1.87 | - | - | -2.20 |
| | XP_019956582.1 | S-adenosylmethionine synthase isoform X1 | - | - | -1.59 | -2.17 | - | - | -2.08 |
| **Other metabolisms GO:0009410** | XP_019958526.1 | 4-aminobutyrate aminotransferase, mitochondrial | x | - | -2.37 | -3.24 | -2.12 | -3.51 | -3.62 |
| | XP_019937416.1 | 78 kda glucose-regulated protein | x | - | - | -1.60 | - | - | - |
| | XP_019956995.1 | Aminoacylase-1 | x | - | - | 2.11 | - | - | - |
| | XP_019954439.1 | Aspartate aminotransferase mitochondrial | x | 2.02 | - | - | - | - | - |
| | XP_019939391.1 | Dynamin-2-like | x | 1.56 | - | - | - | - | - |
| | XP_019936595.1 | Hypoxanthine-guanine phosphoribosyltransferase | x | 2.50 | - | - | - | - | - |
| | XP_019945367.1 | Histamine N-methyltransferase-like | x | - | - | - | 2.14 | - | - |
| | XP_019964208.1 | Primary amine oxidase liver isozyme-like | x | - | - | 1.54 | - | - | - |
| | XP_019954909.1 | Proliferating cell nuclear antigen | x | - | 2.00 | - | - | - | - |
| | XP_019949439.1 | Serine/threonine-protein phosphatase 2A 55 kda regulatory subunit B alpha isoform | x | 1.79 | - | - | - | -1.57 | - |

**Table 6.** List of dysregulated proteins related to urea cycle in *P. flesus* liver in Gouessant, Guillec, Flèche, Quillimadec, Aber Wrac'h and Douffine *vs* Aven.

| | Accession | Name | GO Term | Gouessant | Guillec | Flèche | Quillimadec | Aber Wrac'h | Douffine |
|---|---|---|---|---|---|---|---|---|---|
| **Urea cycle GO:0000050** | XP_019952912.1 | Argininosuccinate lyase isoform X1 | x | - | - | - | 1.57 | - | 1.50 |
| | XP_019937290.1 | Argininosuccinate synthase | x | - | - | -3.80 | 2.53 | -2.38 | - |
| | XP_019966168.1 | CAD protein partial | x | - | - | - | - | - | 1.59 |
| | XP_019954439.1 | Aspartate aminotransferase, mitochondrial | - | 2.02 | - | - | - | - | - |
| | XP_019945774.1 | Aspartate-tRNA ligase cytoplasmic | - | 1.85 | - | - | - | - | - |

**Table 7.** List of dysregulated proteins related to thermal stress in *P. flesus* liver in Gouessant, Guillec, Flèche, Quillimadec, Aber Wrac'h and Douffine *vs* Aven.

| | Accession | Name | GO Term | Gouessant | Guillec | Flèche | Quillimadec | Aber Wrac'h | Douffine |
|---|---|---|---|---|---|---|---|---|---|
| **Response to thermal stress GO:0009408** | XP_019950692.1 | 14-3-3 protein epsilon isoform X1 | x | 1.93 | - | - | - | - | - |
| | XP_019941260.1 | Creatine kinase M-type | x | - | - | - | - | 2.17 | - |
| | XP_019945309.1 | DNAJ homolog subfamily C member 3 | - | - | - | -2.17 | 2.38 | - | - |
| | XP_019953119.1 | Eukaryotic translation initiation factor 2 subunit 1 | x | 2.33 | - | - | 1.54 | - | - |
| | XP_019937416.1 | Glucose-regulated protein 78 kDa | - | - | - | -1.60 | - | - | - |
| | XP_019958780.1 | Heat shock protein 70 kDa protein 4 | x | - | - | - | 1.51 | - | - |
| | XP_019940529.1 | Heat shock protein 70 kDa protein 4L | - | - | - | - | - | - | 1.50 |
| | XP_019961405.1 | Heat shock protein 10 kDa, mitochondrial | - | - | - | -1.72 | - | -1.63 | - |
| | XP_019961403.1 | Heat shock protein 60 kDa, mitochondrial | x | 1.54 | - | - | - | - | - |
| | XP_019957327.1 | Heat shock protein 75 kDa, mitochondrial-like | - | - | 1.71 | - | 1.57 | - | - |
| | XP_019967007.1 | Plasminogen, partial | x | 1.61 | - | - | - | - | - |
| | XP_019941387.1 | Stress-induced-phosphoprotein 1 | - | - | -1.88 | - | - | -1.76 | -1.67 |
| | XP_019952975.1 | Translation initiation factor eIF-2B subunit delta | x | - | - | - | - | 1.50 | - |

**Table 8.** List of dysregulated proteins related to hypoxia in *P. flesus* liver in Gouessant, Guillec, Flèche, Quillimadec, Aber Wrac'h and Douffine *vs* Aven.

| | Accession | Name | GO Term | Gouessant | Guillec | Flèche | Quillimadec | Aber Wrac'h | Douffine |
|---|---|---|---|---|---|---|---|---|---|
| **Response to hypoxia GO:0001666** | XP_019947567.1 | Alpha-1-antitrypsin homolog | x | 2.04 | - | - | - | - | - |
| | XP_019960693.1 | Delta-aminolevulinic acid dehydratase, partial | x | - | - | - | -2.83 | - | - |
| | XP_019955757.1 | Glycogen phosphorylase, muscle form | x | - | 1.54 | 1.87 | - | - | 1.57 |
| | XP_019941808.1 | Glycogen phosphorylase, muscle form-like | x | - | - | 1.83 | - | - | - |
| | XP_019941425.1 | Hypoxia up-regulated protein 1 | x | - | -1.62 | -2.29 | 1.73 | - | - |
| | XP_019957754.1 | L-lactate dehydrogenase A chain | x | -2.11 | - | - | - | - | - |
| | XP_019936938.1 | Prohibitin-2 isoform X1 | x | 1.81 | - | - | - | - | - |
| | XP_019946232.1 | Protein NDRG1 | x | 3.41 | - | - | - | - | - |
| | XP_019953062.1 | Protein-tyrosine kinase 2-beta-like | x | -3.00 | - | - | - | - | - |

**Table 9.** List of dysregulated proteins related to lipid metabolism in *P. flesus* liver in Gouessant, Guillec, Flèche, Quillimadec, Aber Wrac'h and Douffine *vs* Aven.

| | Accession | Name | Gouessant | Guillec | Flèche | Quillimadec | Aber Wrac'h | Douffine |
|---|---|---|---|---|---|---|---|---|
| **Fatty acid metabolic process GO:0006631** | XP_019941296.1 | Acetyl-CoA carboxylase 1 isoform X3 | - | - | - | -4.05 | -4.50 | - |
| | XP_019938598.1 | ATP-citrate synthase-like | - | - | - | - | -2.20 | - |
| | XP_019959772.1 | Bile salt-activated lipase-like | 1.88 | - | - | - | - | - |
| | XP_019934831.1 | Dihydrolipoyl dehydrogenase, mitochondrial | 1.53 | - | - | - | - | - |
| | XP_019959379.1 | Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial | 2.53 | - | - | - | - | - |
| | XP_019936921.1 | Elongation of very long chain fatty acids protein 1-like | 1.78 | - | - | - | - | - |
| | XP_019963310.1 | Fatty acid synthase-like | - | -1.79 | - | -2.-11 | -2.84 | - |
| | XP_019938362.1 | Fatty acid synthase-like | - | - | - | -1.57 | -1.90 | - |
| | XP_019942157.1 | Glutathione peroxidase 1-like | 3.19 | - | - | - | - | - |
| | XP_019958509.1 | Hydroxyacid oxidase 1 | - | - | - | - | 1.50 | - |
| | XP_019950392.1 | Long-chain-fatty-acid--CoA ligase 3 | - | - | - | -2.60 | -2.60 | -2.17 |
| | XP_019941733.1 | LOW QUALITY PROTEIN: long-chain-fatty-acid--CoA ligase 6 | - | - | - | - | -2.00 | - |
| | XP_019967376.1 | Threonine synthase-like 2, partial | - | - | - | - | - | 1.80 |
| | XP_019962064.1 | Thromboxane-A synthase | - | - | -2.29 | - | -3.90 | -2.29 |
| | XP_019962483.1 | Thromboxane-A synthase-like, partial | - | - | | - | -1.67 | - |
| **Fatty acid Oxidation GO:0006631 and GO:0019395** | XP_019933793.1 | 2,4-dienoyl-CoA reductase, mitochondrial-like, partial | -2.00 | - | - | - | - | - |
| | XP_019954722.1 | 3-ketoacyl-CoA thiolase, mitochondrial | - | - | 3.00 | - | - | - |
| | XP_019952981.1 | Acetyl-CoA acetyltransferase, cytosolic | - | - | - | - | - | 1.82 |
| | XP_019956972.1 | ATP-binding cassette sub-family D member 3 | - | 2.00 | - | - | - | - |
| | XP_019949074.1 | Electron transfer flavoprotein subunit beta | - | - | - | - | - | -1.64 |
| | XP_019959379.1 | Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial | 2.53 | - | - | - | - | - |
| | XP_019946456.1 | Fatty acid-binding protein, heart-like | - | - | 2.18 | - | - | 2.82 |
| | XP_019958509.1 | Hydroxyacid oxidase 1 | - | - | - | - | 1.50 | - |
| | XP_019956691.1 | Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial | 1.63 | - | 1.52 | - | - | 1.52 |
| | XP_019940771.1 | Isovaleryl-CoA dehydrogenase, mitochondrial | - | -2.27 | - | - | - | - |
| | XP_019938084.1 | Methylglutaconyl-CoA hydratase, mitochondrial | -2.18 | - | - | - | - | - |
| | XP_019939462.1 | Peroxisomal acyl-coenzyme A oxidase 1 isoform X1 | - | - | - | - | 1.56 | - |
| | XP_019945842.1 | Peroxisomal bifunctional enzyme | - | - | - | -1.60 | - | - |
| | XP_019935560.1 | Very long-chain acyl-CoA synthetase-like | 2.15 | - | - | - | - | - |
| | XP_019934607.1 | Very long-chain acyl-CoA synthetase-like | - | 1.66 | - | 1.86 | 1.66 | 1.58 |
| | XP_019934608.1 | Very long-chain acyl-CoA synthetase-like | - | 1.69 | 1.91 | - | 1.78 | - |
| | XP_019951247.1 | Very long-chain specific acyl-CoA dehydrogenase, mitochondrial | - | - | 2.14 | - | - | 2.00 |

**Table 10.** List of dysregulated proteins related to immune system and complement in *P. flesus* liver in Gouessant, Guillec, Flèche, Quillimadec, Aber Wrac'h and Douffine *vs* Aven.

| | Accession | Name | Gouessant | Guillec | Flèche | Quillimadec | Aber Wrac'h | Douffine |
|---|---|---|---|---|---|---|---|---|
| | XP_019940057.1 | 26S proteasome non-atpase regulatory subunit 12 | - | - | - | 1.55 | -1.82 | - |
| | XP_019933970.1 | Annexin A2 | 2.41 | - | - | - | - | - |
| | XP_019938598.1 | ATP-citrate synthase-like | - | - | - | - | -2.2 | - |
| | XP_019935573.1 | Bifunctio-l glutamate/proline--tr- ligase isoform X1 | - | - | - | 1.58 | - | - |
| | XP_019935873.1 | Cathepsin B-like | - | - | 2.18 | - | - | 1.82 |
| | XP_019954440.1 | Coatomer subunit beta | - | - | -1.83 | - | - | - |
| | XP_019939804.1 | Complement C3-like | - | -1.81 | - | - | -2 | -1.61 |
| | XP_019940984.1 | Complement factor B-like | - | -1.56 | - | - | -1.92 | -1.56 |
| | XP_019952113.1 | Copine-3-like | - | - | -1.5 | -2.45 | - | -1.8 |
| | XP_019943008.1 | Copine-3-like isoform X1 | -1.73 | - | - | - | - | - |
| | XP_019953565.1 | Cytoplasmic dynein 1 heavy chain 1 | - | - | -1.76 | - | - | - |
| | XP_019962518.1 | Desmoplakin isoform X1 | - | - | - | -4.33 | - | -5.2 |
| | XP_019950815.1 | Dipeptidyl peptidase 1 | - | - | - | 1.6 | - | - |
| | XP_019965773.1 | Elongation factor 2 | 3.14 | - | - | - | - | - |
| | XP_019934932.1 | F-actin-capping protein subunit alpha-2 | 5.25 | - | - | - | - | - |
| | XP_019968127.1 | Filamin-B-like | -2.21 | - | - | - | - | - |
| | XP_019968923.1 | Filamin-B-like, partial | - | - | -1.86 | - | - | - |
| | XP_019953732.1 | Glycogen phosphorylase, liver form | - | - | 1.71 | - | - | - |
| | XP_019955013.1 | GTP cyclohydrolase 1-like | 1.67 | - | - | - | 1.81 | 1.51 |
| | XP_019965662.1 | Histone H2B 1/2 | 2.13 | - | - | -1.53 | - | - |
| **Complement activation GO:0006955 GO:0006956** | XP_019936595.1 | Hypoxanthine-guanine phosphoribosyltransferase | 2.5 | - | - | - | - | - |
| | XP_019933904.1 | Importin subunit beta-1-like, partial | - | - | - | 1.71 | - | - |
| | XP_019965427.1 | Isocitrate dehydrogenase [NADP] cytoplasmic-like | - | - | - | - | - | 1.57 |
| | XP_019968933.1 | Isocitrate dehydrogenase [NADP] cytoplasmic-like | - | 1.73 | 1.5 | - | - | 1.76 |
| | XP_019939583.1 | Keratin, type I cytoskeletal 13-like | - | - | - | -2.25 | - | - |
| | XP_019939584.1 | Keratin, type I cytoskeletal 13-like | - | - | - | -2.63 | - | - |
| | XP_019966124.1 | Kinesin-1 heavy chain | 1.61 | - | -2.2 | - | -1.83 | -1.83 |
| | XP_019961736.1 | Leukotriene A-4 hydrolase | - | - | - | 1.89 | - | - |
| | XP_019954492.1 | Lysine-tRNA ligase isoform X1 | 1.61 | - | - | - | - | - |
| | XP_019963864.1 | Major vault protein-like | - | - | - | - | -1.63 | - |
| | XP_019969345.1 | Major vault protein-like, partial | - | - | - | - | -2.05 | - |
| | XP_019969569.1 | Major vault protein-like, partial | - | - | - | - | -2.08 | - |
| | XP_019942688.1 | Methyltransferase-like protein 7A | 1.58 | - | - | - | - | - |
| | XP_019956957.1 | Phosphoglucomutase-2 | - | 1.95 | 1.55 | - | - | 1.5 |
| | XP_019953758.1 | Plasma alpha-L-fucosidase | - | - | - | - | 1.67 | - |
| | XP_019968985.1 | Plastin-2, partial | - | - | - | - | -2.89 | - |
| | XP_019954173.1 | Proteasome subunit beta type-1 | 2.25 | - | - | - | - | - |
| | XP_019953062.1 | Protein-tyrosine kinase 2-beta-like | -3 | - | - | - | - | - |
| | XP_019941652.1 | T-complex protein 1 subunit theta isoform X1 | 2.14 | - | - | - | - | - |
| | XP_019942989.1 | Thioredoxin domain-containing protein 5 | - | -2.14 | -1.76 | - | - | -1.67 |
| | XP_019956568.1 | Transitional endoplasmic reticulum atpase | - | - | - | 1.52 | - | - |
| | XP_019967815.1 | Triokinase/FMN cyclase | 1.98 | - | - | - | -2.13 | - |
| | XP_019952833.1 | Vitronectin-like | - | -2.33 | -1.91 | -2.1 | - | - |

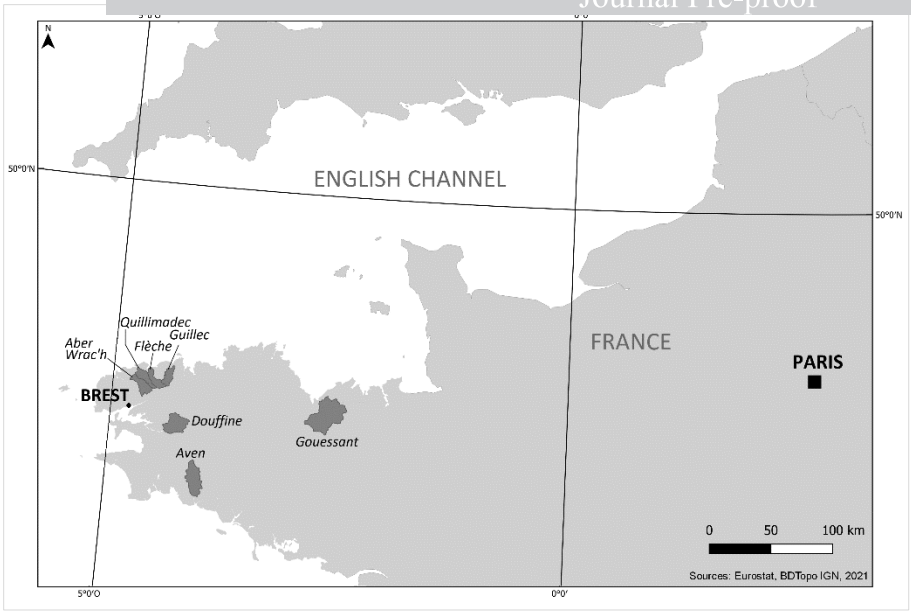


**Figure 1.**

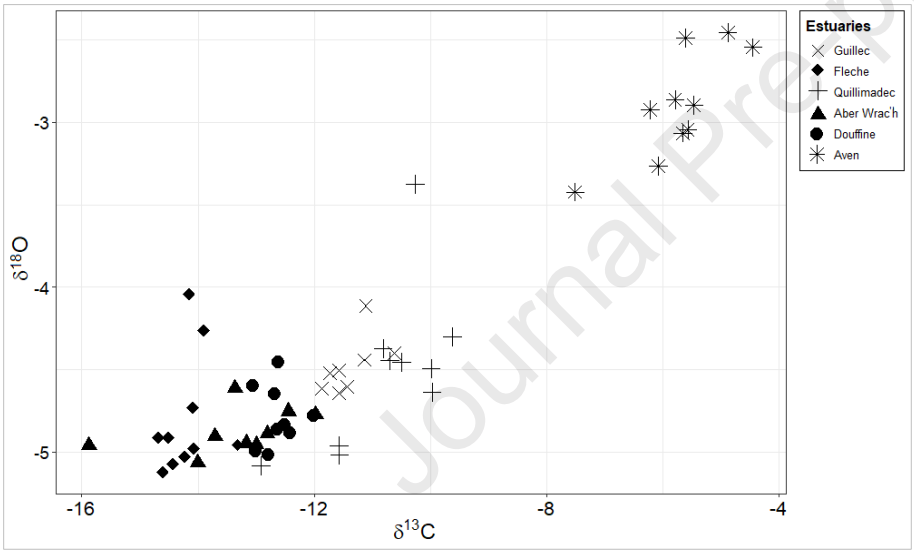


**Figure 2.**

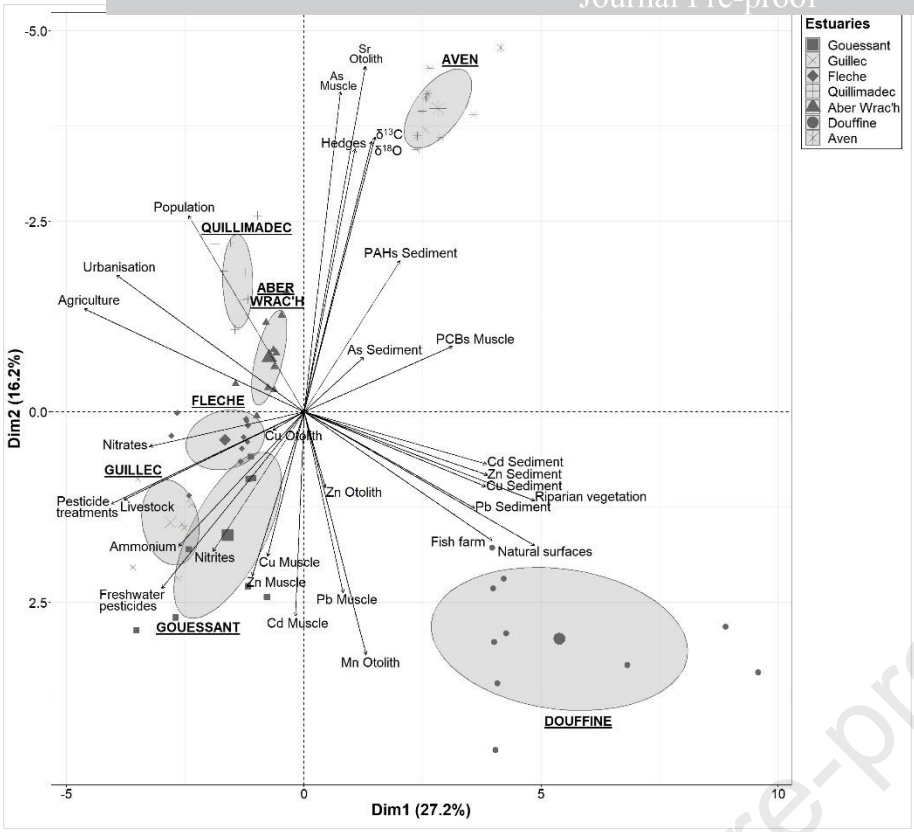


**Figure 3.**

**Figure legends:**

**Figure 1.** Location of the studied catchments and sampled estuaries (Gouessant, Guillec, Flèche, Quillimadec, Aber Wrac'h, Douffine and Aven) and main city (Brest).

**Figure 2.** Stable carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotopes in *P. flesus* otoliths from Guillec, Flèche, Quillimadec, Aber Wrac'h, Douffine and Aven estuaries. **Measurements could not be performed on the flounders from Gouessant because few individuals were captured and the otoliths were used for trace element analysis.*

**Figure 3.** Principal component analysis (axes 1 and 2). Distribution of 31 variables on the correlation circle and individuals on the factorial plan in Gouessant, Guillec, Flèche, Quillimadec, Aber Wrac'h, Douffine and Aven Flounder populations.

* Geographical metrics including land use and landscape patterns were assessed

* Contrasted levels of nitrogen and pesticides were detected in the seven hydrosystems

* Proteomics sharply reflected metabolic alterations of fish submitted to multistress

* Fish showed detoxification, hypoxia response, alterations of urea cycle and immunity

* The major environmental stressors were identified within each hydrosystem

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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