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Histopathological lesions and DNA adducts in the liver of European flounder (*Platichthys flesus*) collected in the Seine estuary versus two reference estuarine systems on the French Atlantic coast

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Abstract:

An epidemiological survey was conducted in the Seine estuary and in two smaller and relatively preserved estuaries on the French Atlantic coast in order to estimate the occurrence of liver lesions in European flounder, *Platichthys flesus*, and also to seek putative risk factors for the recorded pathologies. Four hundred and seventy-eight fish of both sexes and of different size ranges were sampled in the three studied areas, 338 of which in the Seine estuary. All fish were examined for histopathological liver lesions, while DNA adducts and otoliths were analyzed on a subsample. Five categories of hepatic lesions were recorded with the following prevalence for the Seine estuary: 36.7 % inflammations, 8 % parasites (mainly encysted nematodes), 6.5 % foci of cellular alteration (FCA), 5.3 % foci of necrosis or regeneration (FNR), and 1.5 % tumors. Inflammation occurrence increased according to age, contrary to parasitic infestations and FCA which were more prevalent in young fish, notably those of <1 year old (group 0). Tumors were only observed in females of more than two winters. Females exhibited a higher prevalence of tumors (3.0 %) and FCA (6.5 %) than males (0 and 2.6 %, respectively). Parasitic and infectious lesions and FNR were equally distributed in males and females. The prevalence of FNR was also shown to vary according to sampling season, with significantly more occurrences of liver necrosis in the fish collected in summer than in spring. Spatial differences were observed with a higher occurrence of encysted parasites in flounders from the upper Seine estuary, while inflammations predominated in flounders living downstream. Temporal trends were also noted, with an increased prevalence of parasitic infestations, inflammations, and FCA in the 2002–2003 period in comparison to the 1996–1997 one. The three flounder populations from

the Seine estuary (Normandy), Ster estuary (Brittany), and Bay of Veys (Normandy) showed different spectra of hepatic lesions. Flounders from the Bay of Veys had relatively few liver lesions as compared to flounders from the two other estuaries. Flounders from the Ster estuary exhibited the highest prevalence of parasites (37.2 %) and inflammations (51.1 %). Finally, FCA and liver tumors occurred at very similar levels in both flounder populations from the Seine and the Ster estuaries. Group 0 flounders inhabiting the upper Seine estuary were more prone to parasitic and pre-neoplastic hepatic lesions and had higher levels of liver DNA adducts than the older ones living downstream. It was postulated that group 0 European flounders may serve as valuable bioindicators for assessing the quality of estuarine waters and the health status of euryhaline fish populations.

Keywords: Epidemiological study ; European flounder ; Seine estuary ; Liver histopathology ; Necrosis ; FCA ; Tumors ; Inflammations ; Parasites ; DNA adducts ; Pollution

Abbreviations:
OSPAR: Oslo-Paris convention on the protection of the marine environment of the North-East Atlantic.
DRZ: diagonal radioactive zone.
EPCV: European College of Veterinary Pathologists

1. Introduction

Estuarine and coastal marine areas play a crucial ecological role as nursery and/or feeding grounds for a large variety of aquatic species, notably fish (Beck et al. 2001). Since nearly 60 % of the human worldwide population lives and works within 100 miles of a coast (Hinrichsen 1998), these areas are even more impacted by human activities and, particularly, by chemicals. It is now currently accepted that sediments from estuaries and coastal areas are major reservoirs for a large variety of persistent organic or inorganic pollutants and secondary

sources of pollution for aquatic ecosystems (Harris et al., 1996). An increase in the occurrence of toxicopathic lesions and/or infectious or parasitic diseases in aquatic organisms chronically exposed to pollutants has extensively been reviewed in recent years (Harmon and Wiley, 2010; Mearns et al., 2011). Increased susceptibility to pathologies can directly or indirectly impair the survival or biotic performances of individual organisms which, in turn, can affect the abundance, age structure, genetic diversity, and the reproduction of wild populations (Marchand et al., 2004; Benejam et al., 2010; Brooks et al., 2012).

The liver of vertebrates, including fish, is the main target for toxicants because of its high vascularization, its high lipid content and its role in the organic xenobiotic biotransformation and metabolism of sex hormones (Hinton et al., 2001). It has been shown that fish exposure to organic and metallic pollutants can lead to a wide range of toxicopathic lesions, including tumors, FCA (foci of cellular alteration) and several non-neoplastic liver lesions (Hinton et al., 2001). In the field, a strong relationship was reported between toxicopathic liver lesions and environmental contamination by persistent organic pollutants including PAH and organochlorinated hydrocarbons (Myers et al., 1998; Harshbarger and Clark, 1990; Myers et al., 2003). For all these reasons, toxicopathic liver lesions in fish liver are considered as sensitive and integrative biomarkers of pollutant exposure (Hinton et al., 2001) and their integration in a pollution monitoring program is now recommended by the OSPAR international organization (SGIMC, 2011).

The European flounder ***Platichthys flesus*** (L.) is a coastal and estuarine flatfish species which is widespread along the European coast, in the North Sea, the Atlantic Ocean and the Mediterranean Sea. Because of their benthic way of life, their bottom-feeding behavior and their relative longevity, flounders are particularly exposed to sediment-trapped pollutants. In addition, this species has been recommended by OSPAR for pollution monitoring in the North-East Atlantic.

Several epidemiological studies focusing on liver pathologies in the European Flounder have already been conducted in the North Sea and the Baltic Sea along the British, German and Dutch coasts (Bogovski et al., 1999; Vethaak, 1992; Koehler, 2004; Vethaak et Wester, 1996; Stentiford et al., 2003; Lang et al., 2006). But no or little data is available for the French Atlantic coast. Furthermore, risk factors for certain liver lesions are not totally well-understood and need further investigation.

In the present study, flounders were collected on the French Atlantic coast, in three different estuaries having specific features in terms of size, hydrological conditions, human pressures and water quality.

The Seine estuary is a large (50 km^2), man-altered, macrotidal ecosystem on the French Atlantic coast. The Seine River catchment covers about 79,000 km^2 and is one of the most urbanized and industrialized area in France with about 25% of the metropolitan population (16 million inhabitants), 40% of the national economic activity and 30% of the national agricultural activity. Due to the high pollutant inputs and a relatively low water flow (250 to 900 mm^3/s), PAHs and PCBs are currently detected at high levels in sediments (Cachot et al., 2006), suspended matters (Cailleaud et al., 2007) and biota (Minier et al., 2006; Rocher et al., 2006; Cailleaud et al., 2007). Furthermore, sediments from the upper Seine estuary were shown to contain potent mutagenic and carcinogenic pollutants (Cachot et al., 2006) and adverse health effects were reported in various invertebrates (Minier et al., 2006; Rocher et al., 2006; Cailleaud et al., 2009) and fish species (Marchand et al., 2004; Gilliers et al., 2006; Cachot et al., 2007; Amara et al., 2009).

The Bay of Veys is a large and shallow estuary of about 37 km^2 located in the western part of the Seine bay (east coast of Cotentin). Three main rivers including the Douve, the Taute and the Vire are connected to this bay. The global river catchment of 3,500 km^2 is poorly

urbanized (about 70 inhabitants/km^2) showing mainly agricultural activity (cattle breeding and dairy farming).

The Ster of Lesconil estuary is a small (0.36 km^2) and pristine area located in Brittany with a water catchment of about 100 km^2. This river receives reduced domestic and agricultural inputs and no industrial waste, and the levels of organic pollutants and metals currently found in mussels and European flounders are low (Marchand et al., 2003; 2004).

The main objective of the present work is to make a first survey of liver pathologies and DNA adduct levels in feral European flounders from these three contrasted estuaries. In addition, this study aims at analyzing, for each of the various flounder liver pathologies, the putative risk factors including biotic traits (age and sex) and abiotic parameters such as localization, season and year of collection.

2. Materials and Methods

2.1 Sampling sites

The epidemiological survey was conducted in three estuaries along the French Atlantic coast: the Seine estuary (Normandy, France), the Bay of Veys (Normandy, France) and the Ster of Lesconil estuary (Brittany, France) (Figure 1A).

The Seine estuary extends over 170 km from Poses to Le Havre (Cap de la Hève). Three distinct areas can be distinguished within the Seine estuary (Figure 1B): the upper Seine estuary from Poses (upstream limit of the dynamic tide) to Caudebec which is the fresh water part of the estuary, the middle Seine estuary from Caudebec to Honfleur with mesohaline water, and the lower Seine estuary from Honfleur to the Seine Bay characterized by salt water and a high tidal range. A fourth area, named Antifer, localized in the Eastern part of the Seine

bay along the Pays de Caux but under the direct influence of the Seine river plume was also sampled (Figure 1B).

2.2 Fish sampling and liver collection

Juvenile and adult European flounders (***Platichthys flesus***, L.) of both sexes were collected by trawling or netting in the course of 15 fishing campaigns between April 1996 and May 2004 (Table 1). Immediately after sampling, alive fish were transferred to tanks filled with recirculated water prior to dissection. Amongst the 1505 captured fish, 478 fish were randomly selected (the first twenty individuals for each sampled sites or all the captured fish if less than twenty) for liver histopathological analysis. The fish had their spinal cord cut and abdominal cavity carefully opened with dissecting scissors, in order to have the integrity of their internal organs preserved. The fish was sized (total body length), sex was determined, and the biggest otolith (the sagittal) was recovered from randomly selected fish for age determination. The whole liver of small fish (up to 110 mm in length) was sampled for histological examination, while for bigger individuals, 1 cm^3 slice encompassing the whole tissue thickness and width was collected using clean razor blades. The liver samples were immediately fixed in 10% formalin buffer solutions. DNA adducts were measured in the liver of flounders collected in the Seine estuary in September and October 1996 and in the Bay of Veys in April 1997. A small piece of liver of about 1 cm^3 was recovered from 10 to 15 individual fish of both genders and within the same size range. Immediately after collection, liver samples were pooled in a clean DNase-free microtube and deep-frozen in liquid nitrogen. Samples were then stored at -80°C prior to analysis.

2.3 Age determination

The biggest otolith (the sagittal) was removed from 87 randomly selected fish of different size and sex which had been caught in the Seine estuary. Otoliths were immersed in water and examined with a stereomicroscope (Leica Wild M8) under transmitted light. Translucent annuli corresponding to winter periods were counted.

2.4 Liver histopathology

Formalin-fixed tissues were dehydrated by transferring them through a series of alcohols of increasing concentrations (80%, 2*1 hour, 35°C, 2 times; 95%, 2*1 hour, 35°C, 2 times) up to 100% alcohol (4*1 hour, 4 times). Next, they were placed into methylcyclohexan which is miscible with both 100% alcohol and paraffin (methylcyclohexan, 3*1 hour, 35°C, 3 times). Then they were put into melted paraffin (3*1 hour, 58°C, 3 times). All these operations were performed using an Automat Tissue-Tek VIP 3000. The samples were then embedded in paraffin wax (Automat Tissue-Tek TEC-5) and sliced with a microtom Reichert-Jung 2030 into 4-µm thick sections. The sections were stained using a routine hematoxyllin-eosin-saffron staining method (Automat Sakura DRS601). A representative section of each sample was examined in a double-blind manner by means of light microscopy by one of the ECVP-pathologists (European college of veterinary pathologists, either authors #2 and/or #3 because of the long-term study), but the critical slides were observed by the two pathologists. All lesions were assigned to five main categories recommended for monitoring the biological effects of contaminants in flatfish species: parasitic lesions, inflammations, necrosis/regeneration foci, foci of cellular alteration and neoplasms (Feist et al., 2004). Melano-macrophage aggregates were counted in ten optical fields at high magnification (x400), and only more than 2 aggregates for ten optical fields were considered as an abnormal accumulation.

2.5 DNA adduct analysis

The livers of 10 to 15 individuals were pooled, and high molecular weight DNA was extracted using the chloroform/isoamyl alcohol method. Livers were homogenized in 0.8 mL of a solution containing NaCl (0.1 M), EDTA (20 mM), and Tris-HCl, pH 8 (50 mM) (SET). One hundred µL of SDS (20%) was added to the homogenate, and following incubation for 10 min at 65°C, 800 µL of potassium acetate (6M, pH5) was added. Then the reaction mixture was kept at 0°C for 30 min. After centrifugation for 25 min at 0°C (10000g), the supernatant was collected and its nucleic acid content was precipitated overnight at -20°C by adding 2 volumes of cold ethanol. DNA pellets were collected, washed once with 1mL of 90% ethanol and dissolved in 500µL of SET (15 min at 37°C). The total extract was mixed with 10µL of a mixture of RNase A (20 mg/mL) and RNase T1 (10 000 U/mL) and incubated for 1 h at 37 °C; this treatment was repeated twice. Samples were then treated with 20 mg/mL of proteinase K for 1 h at 37 °C. After digestion, 500µL of Rotiphenol was added. The mixture was then moderately shaken for 20 min at room temperature and centrifuged for 15min at 15°C (10000g). The aqueous phase was collected after two extractions. After a final extraction with one volume of chloroform/isoamyl alcohol (24:1), the aqueous phase was collected and 50µL of sodium acetate (3M, pH 6) was added. The DNA was precipitated by the addition of two volumes of cold ethanol overnight at -20°C followed by centrifugation at 10000g for 30 min. The DNA pellet was washed four times with 90% ethanol. The purity of the DNA was checked by recording UV spectra between 220 and 320 nm.

DNA adducts were measured using the ^{32}P-postlabeling method with Nuclease P1 treatment as described by Reddy and Randerath (1986) with minor modifications as follow. DNA (4µg) was digested at 37°C for 4h with micrococcal nuclease (500mU), spleen phosphodiesterase (105mU) buffered with sodium succinate (200mM), and calcium chloride (100mM, pH 6). The digested DNA was then treated with a mixture containing nuclease P1 (4mg/mL), $ZnCl_2$

(1mM) and sodium acetate (0.5M, pH 5) at 37°C for 45min. The reaction was stopped by adding 3 µL of Tris base. The DNA adducts were labeled as follows. Five µL of the reaction mixture containing 2 µL of bicine buffer [Bicine (800 µM), dithiothreitol (400 mM), $MgCl_2$ (400 mM), and spermidine (400 mM) adjusted to pH 9.8 with NaOH], 9.6U of polynucleotide kinase T4, and 100 µCi of [^{32}P]ATP (specific activity 6000Ci/mmol) was added to the NP1 digest and incubated at 37°C for 45 min. Normal nucleotides, pyrophosphate, and excess ATP were removed by chromatography on PEI/cellulose TLC plates in 2.3M NaH_2PO_4 buffer, pH 5.7, overnight. The origin areas containing labeled adducted nucleotides were cut out and transferred to another PEI/cellulose TLC plate, which was run in 5.3M lithium formate and 8.5M urea (pH 3.5) for 3 h. A further migration was performed after turning the plate 90° anticlockwise in 1 M LiCl, 0.5M Tris and 8M urea (pH 8) for 2 h. Finally, the chromatogram was washed in the same direction in 1.7M NaH_2PO_4, pH 6, for 2 h (D4). Autoradiography was carried out at -80 °C for 48 h in the presence of an intensifying screen. Radioactive spots were detected by autoradiography on Kodak super X-Ray film.

Quantification of DNA adducts was obtained by storage phosphor imaging techniques. The screens were scanned using a Typhoon 9210 (Amersham). The software program used to process the data was ImageQuant (version 5.0). After background subtraction, the levels of DNA adducts were expressed as relative adduct labeling (RAL) for 10^9 nucleotides. The sensitivity of the method allows detection of B[a]P adduct as low as 0.1 nucleotide/ 10^{10} nucleotides.

2.6 Statistical analysis

All statistics were performed with the SAS System release 9.3 (SAS Institute Inc., Cary, NC, USA). Categorical data was tested using a Fisher's exact test or a Cochran-Armitage trend test. Bonferroni's correction was applied for pairwise comparisons. The age and length

relationship was investigated using linear regressions (Proc reg). Logistic regressions were used in order to elucidate the implication of period, season, sex or geographic localization on histological responses (Proc logistic). A p-value below 0.05 was considered as significant.

3. Results

3.1 Age of the sampled fish

Age determination was performed by counting growth rings on otoliths from 87 flounders collected in the Seine estuary. Most collected fish had one to four winters and only 3 out of 87 (3.4%) were much older (Figure 2).

In the first three years of fish life (2 winters), a clear age-dependent increase in total body length was observed (Figure 2). For older fish (3 winters and more), body length was no more related to age. In the present study, fish were ranked into four groups according to body length and estimated age. Group 0 included fish up to 110 mm in length and less than one year old. Group 1 included fish sized 115 up to 200 mm and aged from one to less than two years old. Group 2 included individuals of 205 up to 300 mm and aged two to less than three years old. Finally, group 3+ included all fish above 300 mm with an expected age of three years and more.

The different groups were not equally distributed throughout the Seine estuary, with group 0 mainly located in the upper Seine estuary, group 1 in the upper and median estuary, group 2 in the median and lower estuary, and finally group 3+ only represented in the lower estuary and in the Seine bay.

None of the flounders sampled in the Bay of Veys and in the Ster estuary belonged to group 0. Because of age-structure differences between the three flounder populations, histopathological data from the Bay of Veys and Ster estuary were only used for inter-site comparisons with the Seine estuary.

3.2 Description of flounder liver pathologies

The liver of the European flounder is composed of parenchymal cells (hepatic cells) and clusters of pancreatic cells along the branches of the portal vein. A hepatic cell has a polygonal body containing a clear spherical nucleus, usually with a single nucleolus. Large quantities of lipids and glycogen are usually observed in the cytoplasm and cause the clear vacuolar appearance of the cell.

Lesions, encountered during the liver pathology assessment were categorized into five main items (Figure 3).

(i) Encysted parasites (mainly nematodes) are either surrounded by inflammatory cells, that are mainly macrophages forming a granuloma, or only by a thin fibrous capsule (Figure 3A). Depending on the sectioning level, the center of the granuloma and the causative parasite were not always observable (Figure 3B). Less frequently, amoeba infections are associated with ill-delineated foci of necrosis with an influx of macrophages (Figure 3D).

(ii) Inflammatory lesions are characterized by the accumulation of inflammatory cells (lymphocytes and macrophages) mainly observed at the vicinity of major blood vessels. Different grades of inflammations were observed, from a few accumulated melano-macrophage aggregates spread in the liver parenchyma to severe diffuse hepatitis. As the melano-macrophage aggregates can occur under normal conditions in flounder as in other fishes, only more than two aggregates for ten optical fields were considered as an abnormal accumulation (Figure 3C). Diffuse inflammations were very rarely observed and all inflammation types, except host reaction to parasites as described in (i), were jointly recorded.

(iii) Foci of necrosis and regeneration (FNR) were jointly recorded because they can both result from cell death following parasitic infestation, bacterial or viral infection and exposure to toxicants. Focal necrosis is characterized by small groups of hepatocytes exhibiting strong

eosinophilic or pale vacuolated cytoplasm and hyperchromatic-condensed nuclei (Figure 3D). On rare occasions, some foci of small hepatocytes having a high mitosis index formed new trabeculae and were identified as regenerative foci.

(iv) Foci of cellular alteration (vacuolated cell foci, clear cell foci, eosinophilic foci, basophilic foci or mixed eosinophilic and basophilic foci) are focal areas exhibiting different staining degrees: some altered staining behaviors can be eosinophilic or basophilic, and some others can be poorly stained (clear cell foci) (Figure 3E, 3F and 3G). These foci do not compress the surrounding parenchyma. Some of these lesions (basophilic foci) are considered as putative pre-neoplastic lesions (Myers et al., 1987).

(v) Tumors were identified as hepatocellular or pancreatic, and as adenoma (Figure 3H) or adenocarcinoma (Figure 3I). Bile duct tumors were not observed herein. Benign as well as malignant tumors are characterized by cellular atypia associated with architectural abnormalities such as tubular, acinar or solid growth patterns. The tumor growth is associated with a compression (benign) or infiltration (malignant) of the surrounding tissue.

3.3 Prevalence of flounder liver pathologies according to age in the Seine estuary

Granulomas with encysted parasites were observed in 8% (27/338) of sampled flounders in the Seine estuary. Parasite prevalence declined significantly according to age group ($p<0.001$, Fisher's exact test), with a higher value in group 0 fish (27%) than in older ones (3.7%, 7.2%, 4.6% for groups 1, 2, 3+ respectively) (Figure 4A).

Nearly 37% of the sampled flounders (124/338) exhibited liver parenchyma inflammations. Inflammation prevalence significantly differed according to age ($p<0.001$, Fisher's exact test) and was globally higher in old fish of three years and more (58.3%) than in younger ones (22.2 to 32.4%) (Figure 4A).

Foci of necrosis and regeneration (FNR) were recorded in 5.3% (18/338) of the sampled flounders. No significant difference was observed according to age group with prevalence varying from 8.1% for group 0 to 4.6% for group 3+ (Figure 4A).

More than 6% (22/338) of all sampled flounders exhibited foci of cellular alteration (FCA). FCA were observed in all groups but with significant prevalence differences according to fish age ($p<0.05$, Fisher's exact test). Indeed, FCA prevalence was at least three times higher in group 0 (18.9%) than in other groups (5.6%, 5.8% and 3.7% for group 1, 2 and 3+ respectively) (Figure 4B).

Hepatic tumors were recorded in 5 out of 338 flounders (1.5%). Adenoma occurrences were recorded but no adenocarcinoma was observed. Although not significant, an increasing trend of tumor prevalence was observed with age from 0% for age groups 0 and 1 to 0.7% and 3.7% for groups 2 and 3+ respectively (Figure 4B).

3.4 Prevalence of flounder liver pathologies according to gender in the Seine estuary

Prevalence of parasitic infestations, inflammations and FNR did not vary significantly according to sex (Figure 5A). Although the differences were not significant, FCA were 2.5 times more frequent in females (6.5%) than in males (2.6%) and tumors were only observed in females (3.0%) (Figure 5B). Moreover, when FCA and tumors were scored together, females appeared significantly more affected (9.5%) than males (2.6%) ($p<0.05$, Fisher's exact test).

3.5 Spatial distribution of flounder liver pathologies in the Seine estuary

Prevalence of parasitic infestation was significantly different ($p<0.001$, Fisher's exact test) according to the sampling sites (Table 2). Indeed, the infestation prevalence that was recorded

in flounders from the upper part of the Seine estuary (18.4%) was significantly higher than in fish collected in the median Seine estuary (2.2%) or Antifer (2.3%). In contrast, prevalence of inflammations, FNR and tumor did not show any spatial difference. Although FCA frequency was particularly high in flounders from the upper Seine estuary (14.3%), no statistical difference was observed between the different sampling sites.

3.6 Seasonal variations of flounder liver pathologies in the Seine estuary

Since flounders were sampled all the year round in the Seine estuary, it was possible to analyze the seasonal variations of flounder liver pathologies. No significant seasonal variation was observed for FCA, tumors, parasitic infestations and inflammations. Only FNR ($p<0.05$, Fisher's exact test) did show seasonal fluctuations (Figure 6A and 6B). In flounders from the Seine estuary, FNR occurred more frequently in the summer (11.3%) than in spring (1.0%) (Fisher's exact test, p=0,004).

3.7 Temporal evolution of flounder liver pathologies in the Seine estuary

The epidemiological survey of liver pathologies was conducted for all the sampled sites, except for the lower Seine estuary, over two distinct periods in 1996-1997 and four years later in 2002-2003. Comparisons of liver pathology profiles between the two sampling periods showed significant differences, including for the 2002-2003 period, a 4-fold increase in parasitic infestations ($p<0.01$, Fisher's exact test), a 2-fold increase in inflammations ($p<0.001$, Fisher's exact test) and a 5-fold increase in FCA ($p<0.01$, Fisher's exact test) (Figure 7A and 7B). In contrast, FNR occurrence did not vary significantly over the two sampling periods ($p\geq0.05$, Fisher's exact test). For tumor prevalence, the temporal decrease

was almost significant (p=0.06, Fisher's exact test) with 3.4% of flounders exhibiting liver tumors in 1996-1997 versus 0% in 2002-2003 (Figure 7B).

3.8 Comparison of liver pathologies between flounder populations from the three studied estuaries

Flounders were sampled over the same period in three estuaries along the French Atlantic coast. An attempt was made to compare the profiles of liver pathologies between the three flounder populations (Table 3). Since the age structure was different in the three studied populations with an underrepresentation of younger fish in the Bay of Veys and Ster estuary, only adult fish of more than 200mm (groups 2 and 3+) were selected for this analysis. Prevalence of parasitic infestations and inflammations was significantly different between the three flounder populations ($p<0.001$, Fisher's exact test in both cases). When recorded together, tumors and FCA occurrence was also almost significantly different between estuaries (p=0.053, Fisher's exact test). Prevalence of infestations was significantly higher ($p<0.001$) in the Ster estuary (37.6%) in comparison to the Seine estuary (5.8%) and the Bay of Veys (6.5%). Flounders from the Ster and the Seine populations were more prone ($p<0.01$) to liver inflammations (51.8% and 40.3% respectively) than individuals from the Bay of Veys (15.2%) (Table 3). Flounders from the Ster and the Seine estuaries in contrast to those from the Bay of Veys exhibited FCA (9.4% and 4.9%, respectively) and tumors (1.2 and 2.1% respectively). For FNR occurrence, no significant difference was noted for the three studied populations.

3.9 Relationship between the different flounder liver pathologies

A strong positive correlation between parasitic infection and inflammation ($p<0.001$, Fisher's exact test) and a negative correlation between inflammation and FNR ($p<0.001$, Fisher' exact test) were observed. In fact 89% of fish from the Seine estuary bearing encysted parasites also exhibited liver inflammation while only 25% of fish with FNR also showed inflammation. In contrast, no obvious relationship was noted between the other liver lesions.

3.10 DNA adducts in flounder liver

DNA adducts were measured using the ^{32}P-postlabelling technique from pooled livers of flounders collected in September 1996 in the Seine estuary and in April 1997 in the Bay of Veys. Due to insufficient data available (two DNA adduct analyses per site), statistical analysis could not be performed. Nevertheless some spatial trends could be observed. Firstly, the total number of DNA adducts varied greatly from 21.1±8.0 RAL/10^9 nucleotides in the Bay of Veys to 149±38.5 RAP/10^9 nucleotides in the upper Seine estuary (Figure 8). Secondly, the DNA adduct profiles were totally different in the Seine estuary and in the Bay of Veys. The total number of individual spots per site varied from 4 up to 13 in the Seine estuary, and reached 43 in the Bay of Veys (Data not shown). In addition, amongst the 43 individual spots detected in the fish from the Bay of Veys, only 18 (42%) were shared with flounders from the Seine estuary. Finally, most of the adducts (65 to 82% of RAL) detected in flounders from the Seine estuary were located within the diagonal radioactive zone (DRZ) (Figure 9) while all the DNA adducts detected in flounders from the Bay of Veys were located outside the DRZ (Data not shown).

Some spatial trends can also be noted for the Seine estuary. A 10-fold decrease of total DNA adducts in fish liver was observed between the upper Seine estuary and Antifer (Figure 8). The total number of individual adducts declined from 13 spots in the upper Seine estuary to 4

spots in the lower estuary and in Antifer, and no DRZ DNA adducts was detected in Antifer (Figure 9).

4 Discussion

In the present paper, pattern and occurrence of liver pathologies were investigated in European flounders from the Seine estuary and from two other reference estuaries on the French Atlantic coast. Implications of various biotic (age, sex) and abiotic (year, season, location) factors in the onset of the different liver pathologies were investigated. Liver DNA adduct levels were also measured to evaluate fish exposure to genotoxic pollutants.

4.1 Infectious and parasitic diseases

The most prevalent lesion type was inflammation of liver parenchyma, observed in nearly 37% of the sampled fish. Inflammation foci and melano-macrophage aggregates were jointly recorded. Both lesions can be induced by multiple stress factors, including at least infectious and parasitic diseases and toxicant exposure (Wolf and Wolfe, 2005). This lesion type was more frequent in older fish but no sex-specific difference was observed. Vethaak and Wester did not show any relationship with fish gender or age (Vethaak and Wester, 1996). This apparent discrepancy could at least partially be explained by the fact that in the latter study only fish with a total length of 200mm or more, i.e. only fish of two winters or more were examined (Vethaak and Wester, 1996). In the present study, inflammations were slightly more frequently observed in late winter. Wethaak and Wester (1996) reported the same pattern with a higher occurrence of inflammatory lesions, including parasitic cysts, at the end of winter in flounders captured in spawning grounds. The authors hypothesized that poor conditions and spawning stress could favor bacterial and/or parasite infections.

A clear site-specific profile was also reported with a higher prevalence of inflammatory lesions in individuals from the Ster estuary than in those from the Seine estuary or the Bay of Veys. Previous studies had already documented considerable variations of inflammation occurrence according to geographical area and sampling period from about 12% in Dutch coastal and estuarine waters (Vethaak and Wester, 1996) to 30% in coastal areas of the Baltic sea (Lang et al., 2006) (Table 4).

The second most prevalent lesion type was the encysted parasite lesion observed in 13% of all sampled flounders (8% in the Seine estuary). Only a few studies have reported parasitic cysts in flounder liver (Vethaak, 1992; Vethaak and Wester, 1996; Lang et al., 2006; Dezfuli et al., 2007). At least one larval and five adult nematode species have already been identified in flounders (El-Darsh and Whitfield, 1999). In contrast to inflammatory lesions, parasitic cysts occurred with a higher prevalence in flounders of group 0. This feature could putatively be explained by an age-dependent sensitivity of European flounder to nematode infestation and also by heterogeneous spatial distribution of the parasite in favor of the oligohaline or mesohaline part of the estuary. Significant spatial and temporal differences were also evidenced with a similar distribution pattern to that of liver inflammations. Indeed, prevalence of parasitic cysts was higher in the Ster estuary than in the Seine estuary or the Bay of Veys. Moreover, encysted parasite prevalence was slightly higher in flounders collected in late winter.

The higher frequency of inflammatory lesions and parasitic cysts in the flounder population inhabiting the Ster is probably not related to a significant chemical stress because pollutant inputs in this estuary are low (Marchand et al., 2003,2004). This result could more likely be explained by the localization of this estuary in the southern part of the flounder distribution area and by a possible thermal stress. This thermal stress could directly affect the immune system or the global health condition of the fish, leading to an increased sensitivity to

infectious agents or parasites. It is noteworthy that relatively high frequencies of flounder liver lesions were detected in the Gulf of Biscay, as compared to English coasts and the North Sea (Laroche et al., 2012).

4.2 Toxicopathic liver lesions

Necrosis and regeneration foci were observed in 4.2% of the sampled fish (5.3% in the Seine estuary). No significant difference according to age or gender was observed. This lesion type did not show any significant inter-site variations within the Seine estuary or between the three studied estuaries. In contrast, a clear seasonality was observed with a drastic increase in FNR prevalence in the summer. Seasonal variations of hepatocellular necrosis have already been documented for winter flounders (***Pleuronectes americanus***) sampled along the US Northeast coast (Johnson et al., 1993). Numerous man-made chemicals are known to induce necrotic lesions in the liver of fish but naturally-occurring toxins such as microcystins can also be potent hepatotoxic agents (Wolf and Wolfe, 2005). It was recently documented that blooms of the cyanobacteria ***Microcystis aeruginosa*** producing the microcystin-LR can occur in estuarine waters in the summer time when surface water temperature is high and the stream flow is low (Lehman et al., 2008). Therefore it cannot be ruled out that the higher occurrence of FNR in the liver of flounders in the summer time could not be due to cyanobacterial blooms.

Liver tumors and FCA were observed in 1.3% and 6.3% of all sampled fish respectively (1.5 and 6.5% in the Seine estuary respectively). Tumors were restricted to females aged two years and more. The differential sensitivity of male and female flounders as regards liver tumors has already been reported (Koehler, 2004; Vethaak and Wester, 1996). Exposure to carcinogens likely takes place early in the life of flounders when larvae migrate to their estuarine habitats (Koehler, 2004). Since all the collected individuals live in the same habitat and feed on the

same preys, they are likely exposed to the similar cocktail of carcinogens. Koehler proposed that endogenous factors such as estrogen overproduction during vitellogenesis may explain the higher susceptibility of female flounder to develop liver cancer. Indeed, tumor promotion activity of 17-β-oestradiol had already been demonstrated in several fish species (Nunez et al., 1989, Cooke and Hinton, 1999).

In the present study, adenoma was the predominant tumor type (1.0%) while adenocarcinoma was only observed in a single flounder (0.2%). In fact, flounders bearing malignant tumors are rarely observed in the field (Vethaak and Wester, 1996) likely because of quick death or predation of sick individuals. Tumors and FCA were recorded at similar occurrences in the liver of flounders inhabiting the Seine estuary as well as other estuaries, and coastal areas in the North Sea and Baltic Sea (Table 4). Prevalence was somewhat higher along the German coast and particularly in the Elbe estuary but only fish over 180 mm were analyzed (Koehler, 2004). It is noteworthy that FCA prevalence in flounders from the Seine estuary was recorded at a higher level in group 0 than in the other age groups. Interestingly, Amara and collaborators (2009) have also reported lower growth (daily otolith increments) and condition factor (Fulton's K) for group 0 flounders from the Seine estuary in comparison to those collected in two clean estuaries of the Eastern English Channel. Group 0 flounders are predominantly located in the oligohaline part of the Seine estuary. It was previously documented that sediments from this area were genotoxic resulting at least partially from a high content of mutagenic and carcinogenic PAHs (Cachot et al., 2006). It was also demonstrated that embryos of Japanese medaka exposed to an organic extract of sediment from the upper Seine estuary had an increased mutation rate and developed FCA and neoplasms (Cachot et al., 2007).

In the present work, DNA adducts were measured at high levels in the liver of juvenile flounders inhabiting the upper Seine estuary. In addition, the majority of those DNA adducts

were located in the DRZ. Several authors have reported that DRZ-related DNA adducts are mainly induced by hydrophobic hydrocarbons including polyaromatic hydrocarbon metabolites (Varanasi et al., 1989; Lyons et al., 1999; 2004). Indeed, DRZ-related DNA adducts have been detected in the liver of fish exposed in controlled laboratory conditions to various hydrophobic genotoxicants, including PAHs (Varanasi et al., 1989; Malmström et al., 2000), and numerous field studies have documented a positive relationship between pollutant exposure, notably to PAHs, and DNA adduct levels in fish liver (Varanasi et al., 1989; Ericson et al., 1998; Rose et al., 2000; Shaw and Connell, 2001; Aas et al., 2001; Myers et al., 2003). DNA adducts are currently considered as good molecular dosimeters of carcinogenic exposure (Shaw and Connell, 2001) and experimental data supports the role of DNA adducts in the initiation of chemical carcinogenesis (Miller and Miller, 1981).

It is thus tempting to build a scenario of exposure for flounders dwelling in the Seine estuary. Juveniles, during their first year of life in the upper Seine estuary, are directly exposed to pro-genotoxic and carcinogenic pollutants such as PAHs originating from contaminated sediments and benthic preys. Chronic exposure to these compounds induces DNA damage and cytotoxic effects which in turn, trigger structural changes in liver parenchyma, including inflammations, necrosis and FCA. Since tumor development is a long lasting process, liver tumors appear much later (at least two years later) when fish migrate to their marine habitat. Vethaak and collaborators demonstrated through a 3-year mesocosm experiment with flounder juveniles exposed to a contaminated dredged spoil that at least 30 months is needed for hepatocellular adenoma development while FCA can emerge within 6 to 12 months (Vethaak et al., 1996).

From this study several conclusions can be drawn regarding the use of flounder as a sentinel species for pollution monitoring in estuarine ecosystems. (i) Flounder juveniles settle in estuaries just after metamorphosis and spend at least their first year of life there. (ii) Group 0 flounders exhibit a high susceptibility to pollutants, which was revealed in the present study

by DNA adduct induction and the development of toxicopathic liver lesions including necrosis and FCA. (iii) Tumor development is a long lasting process which does not allow reliable measurement of site-specific pollution. Therefore, the measurement of biochemical and histological markers in flounders from group 0 could represent a valuable approach for pollution biomonitoring and fish health assessment in estuarine areas.

4.3 Conclusion

It was demonstrated herein that flounder liver lesions show high spatial and temporal variations which can be related to various biotic (age, sex, etc.) and abiotic (season, location, pollution, etc.) risk factors. Interestingly, group 0 flounders inhabiting the upper Seine estuary faced a higher prevalence of encysted parasites, and foci of cellular alteration than older ones living in the lower part of the Seine estuary. This result likely reflects differences in the sensitivity of flounders to parasites, pathogens and chemicals, according to age, but also different levels of stress according to season and location. Because of their high sensitivity to multiple stress factors and to their first-year of growth exclusively in estuarine waters, European flounders from group 0 represent valuable bio-indicators for pollution and fish population health monitoring in estuarine ecosystems.

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Figure captions

Figure 1: Maps showing the geographical position of the different sampling sites. Localization of the three sampled estuaries (A) and the different sampling areas within the Seine estuary (B).

Figure 2: Age-length relationship for European flounder specimens collected in the Seine estuary.

Figure 3: Histological presentation of European flounder liver lesions. The liver is composed of parenchymal cells (a cell that has a polygonal body containing a clear spherical nucleus and clear appearance of the cytoplasm) and clusters of pancreatic cells along the branches of the portal vein (large dichromatic cells). (A) Parasitic cyst at the vicinity of a pancreatic islet surrounded by a thin rim of fibrous tissue corresponding to encapsulation (arrow). (B) Two well-delineated granulomata slightly compressing the surrounding liver parenchyma (arrows) are composed of large ill-delineated slightly basophilic mononuclear cells characteristic of macrophages. (C) Melano-macrophagic aggregates: randomly scattered in the liver parenchyma, they are composed of large fainted, rarely pigmented cells without encapsulation. (D) A large focus of liquefactive necrosis with cellular debris and numerous amoeba (not shown at this magnification). (E-G) Alteration foci are observed in the parenchyma without compression and are composed of hepatic cells with modifications of their trabecular organization, their size and their cytoplasmic staining: (E) Vacuolated cell alteration focus, ill-delineated focus made of large macro-vacuolated cells (arrow); (F) eosinophilic alteration focus: well-delineated focus made of hypereosinophilic cells; (G) Small basophilic alteration focus: ill-delineated focus made of small hyperbasophilic cells (arrow). (H) Pancreatic adenoma with papillary organization of tumoral cells, well-delineated, encapsulated and compressing the surrounding area of the pancreatic parenchyma. (I) Large infiltrative hepatocellular carcinoma with tubulo-papillar differentiation and a fibrous stroma,

totally replacing liver parenchyma. Hematoxylin-Eosin-Saffron. (A, B, H, I) Bar = 100 µm; (C-G) Bar = 50 µm.

Figure 4: Prevalence (%) of liver pathologies in European flounders collected in the Seine estuary according to age group. (A) Parasitic, inflammatory, necrotic and regenerative lesions. (B) Pre-neoplastic and neoplastic lesions. Significant differences between age groups are indicated by different letters ($p<0.05$, Fisher's exact test with Bonferroni's correction).

Figure 5: Profile of liver pathologies in European flounders from the Seine estuary according to gender. (A) Parasitic, inflammatory, necrotic and regenerative lesions. (B) Pre-neoplastic and neoplastic lesions. Significant differences between genders are indicated by different letters ($p<0.05$, Fisher's exact test with Bonferroni's correction).

Figure 6: Seasonal variations of liver pathologies in European flounders from the Seine estuary. (A) Parasitic, inflammatory, necrotic and regenerative lesions. (B) Pre-neoplastic and neoplastic lesions. Significant differences between seasons are indicated by different letters ($p<0.05$, Fisher's exact test with Bonferroni's correction).

Figure 7: Temporal evolution of liver pathologies in European flounder from the Seine estuary. (A) Parasitic, inflammatory, necrotic and regenerative lesions. (B) Pre-neoplastic and neoplastic lesions. Significant differences between sampling sites are indicated by different letters ($p<0.05$, Fisher's exact test with Bonferroni's correction).

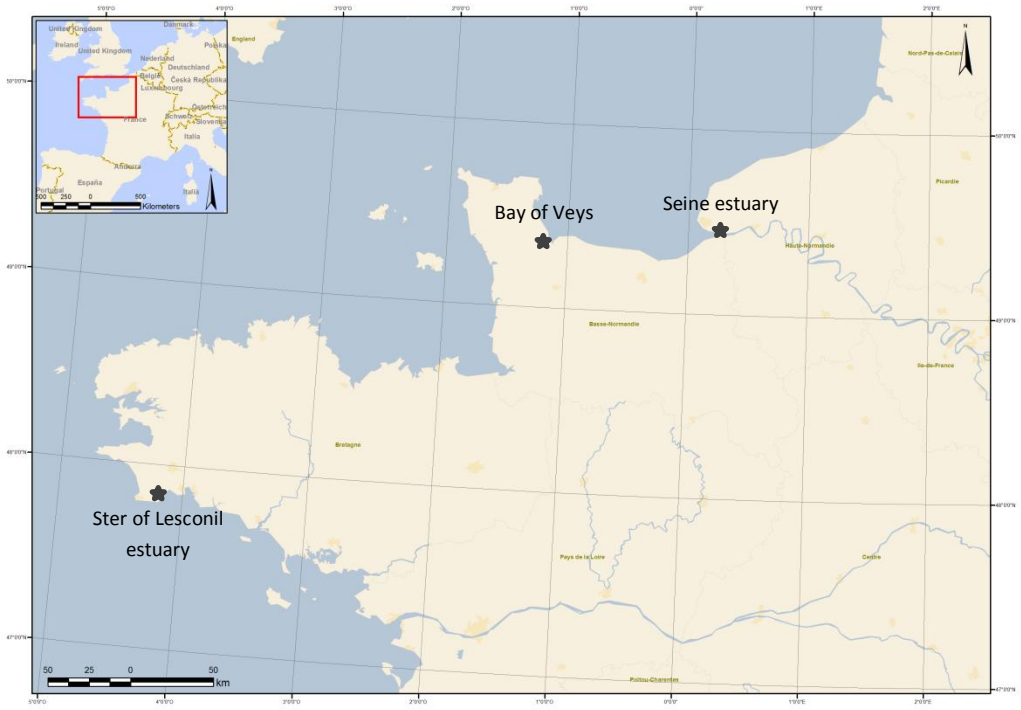
Figure 8: DNA adduct levels in the liver of European flounders collected in the Seine estuary (September and October 1996) and in the Bay of Veys (April 1997). Data are expressed as relative adduct labeling per 10^9 nucleotides (Mean ± SD, N=2)

Figure 9: Representative autoradiograms obtained for European flounders collected in the upper Seine estuary (A) or in Antifer (B). In the first autoradiogram, DNA adducts are

predominantly located within the diagonal radioactive zone (DRZ) while in the second only individual spots outside the DRZ are readily visible.

Figure 1

A


Bay of Veys
Seine estuary
Ster of Lesconil
estuary

B

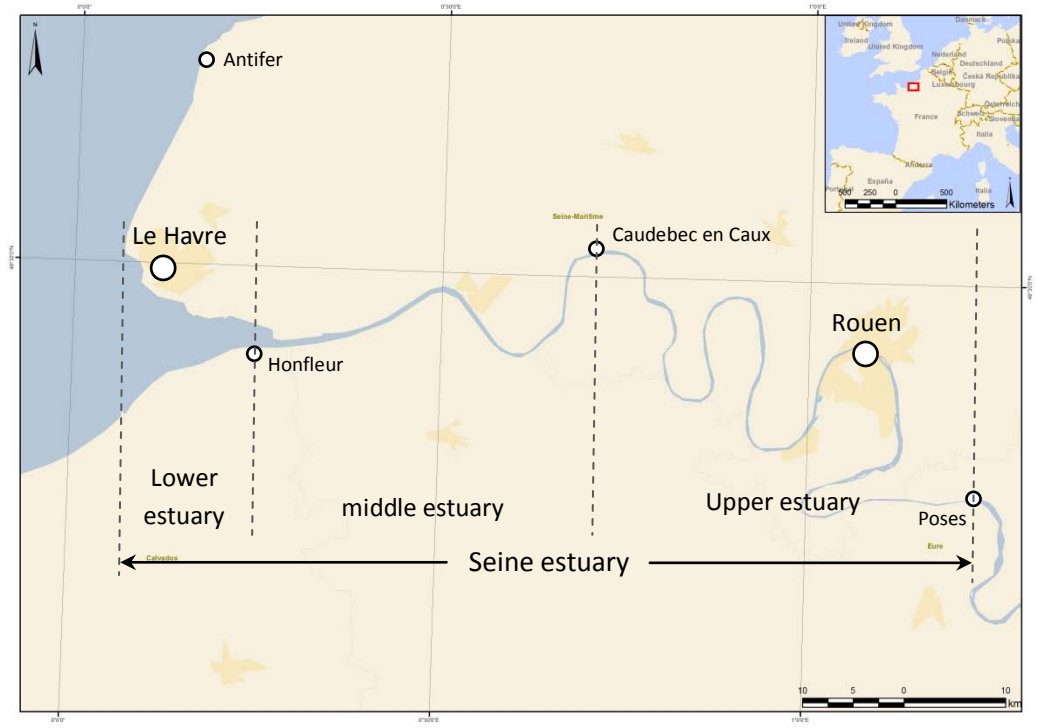

Antifer
Le Havre
Caudebec en Caux
Honfleur
Rouen
Lower
estuary
middle estuary
Upper estuary
Poses
Seine estuary

Figure 2

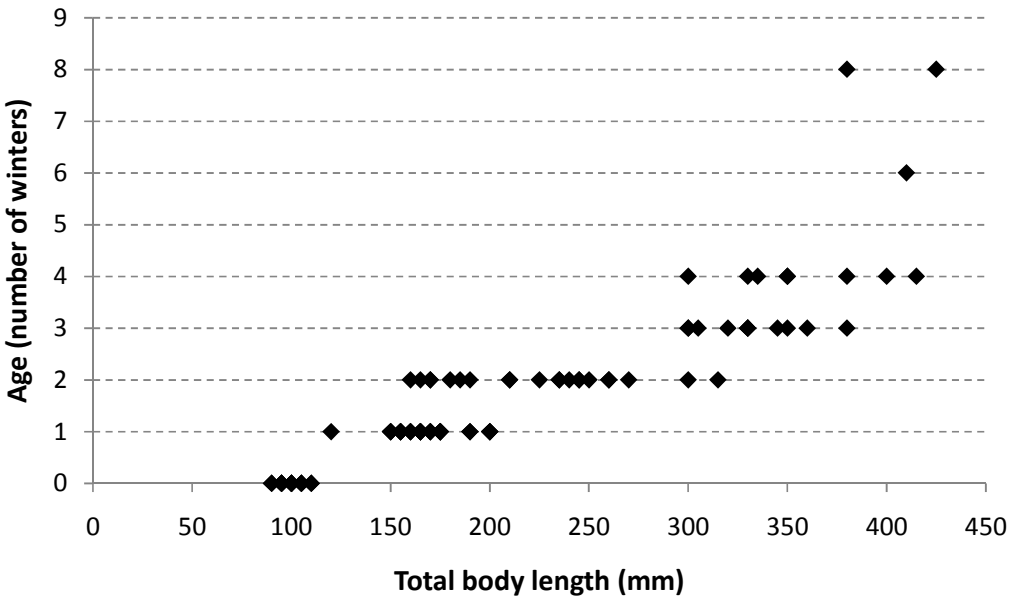
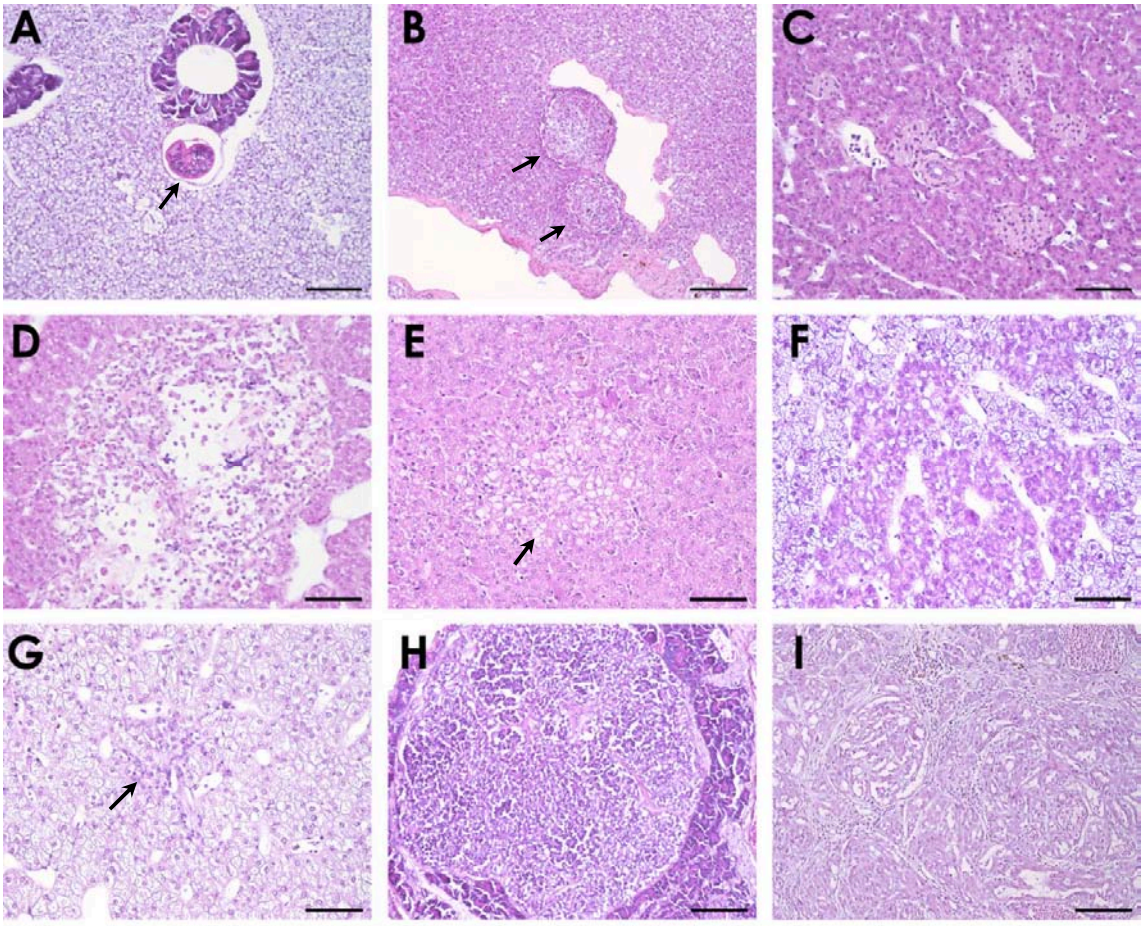
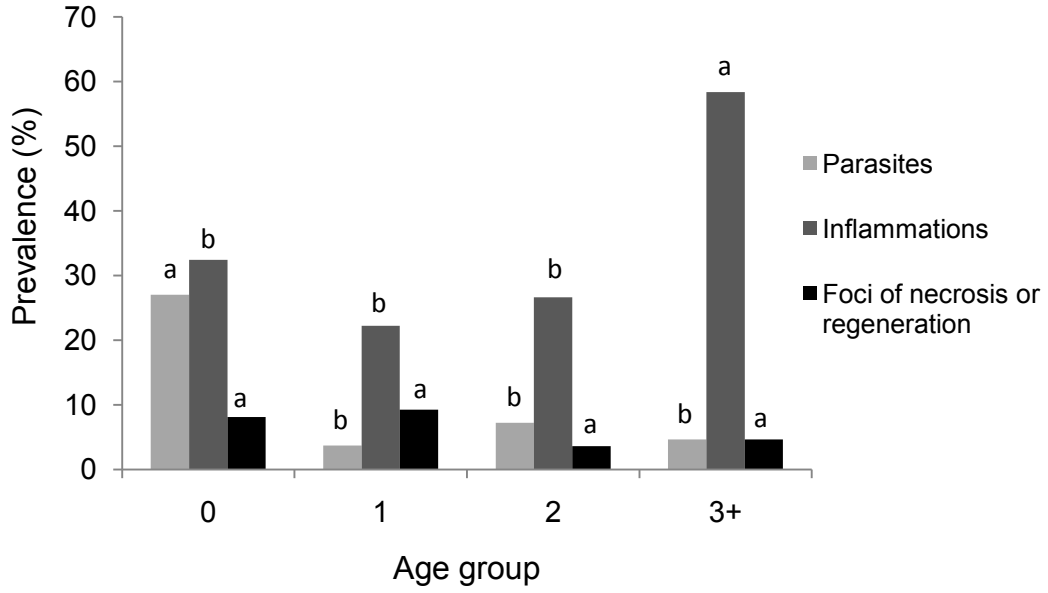


Figure 2

Figure 3



A


70
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50
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10
0
Prevalence (%)
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a
b
b
a
b
b
a
b
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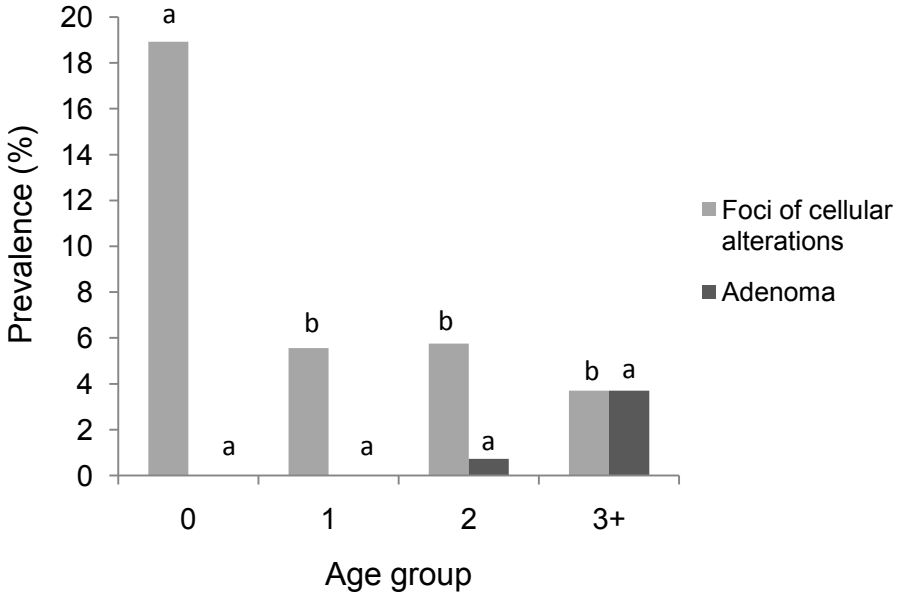
a
0
1
2
3+
Age group
Parasites
Inflammations
Foci of necrosis or regeneration

B


20
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Prevalence (%)
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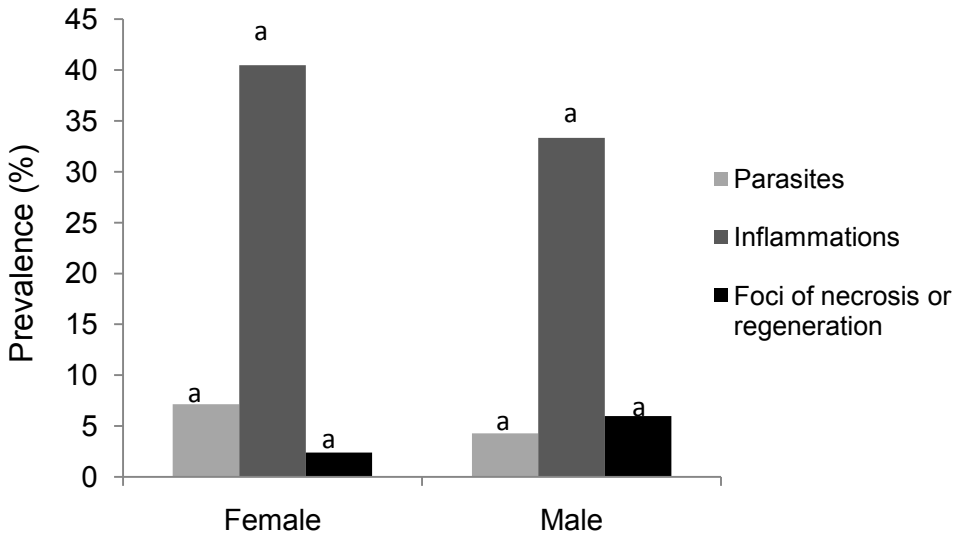
Age group
Foci of cellular alterations
Adenoma

Figure 4

A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20
15
10
5
0
Prevalence (%)
a
a
a
a
a
a
Female
Male
Parasites
Inflammations
Foci of necrosis or regeneration

B

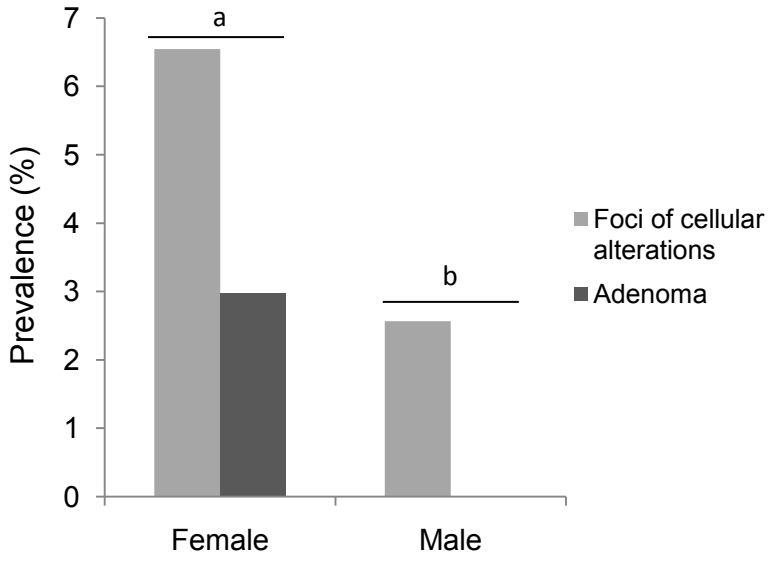
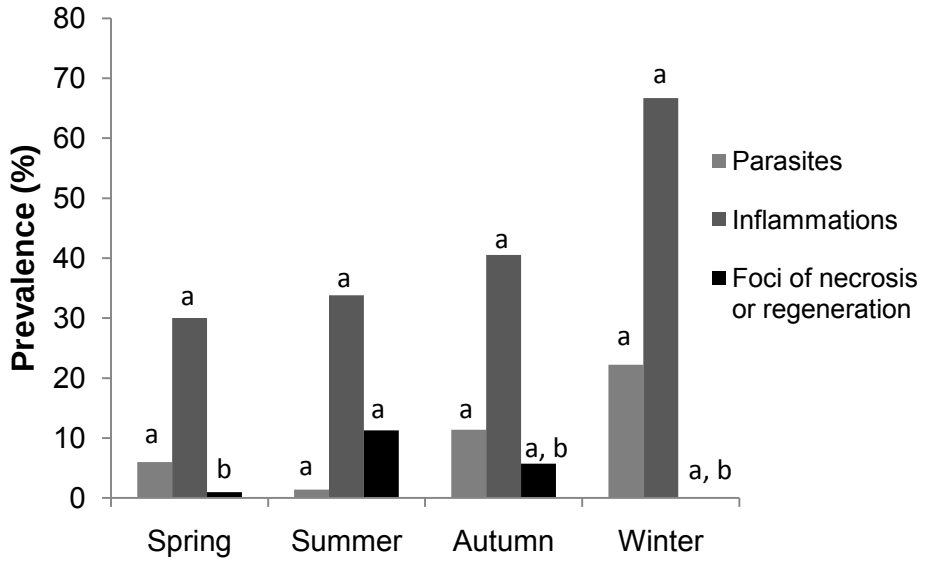

7
6
5
4
3
2
1
0
Prevalence (%)
a
b
Female
Male
Foci of cellular alterations
Adenoma

Figure 5

Figure 6

A



B

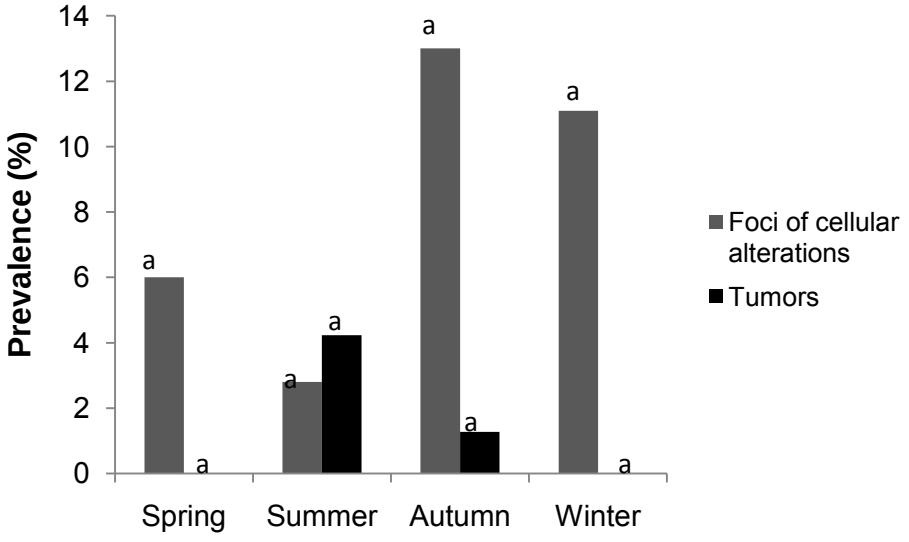
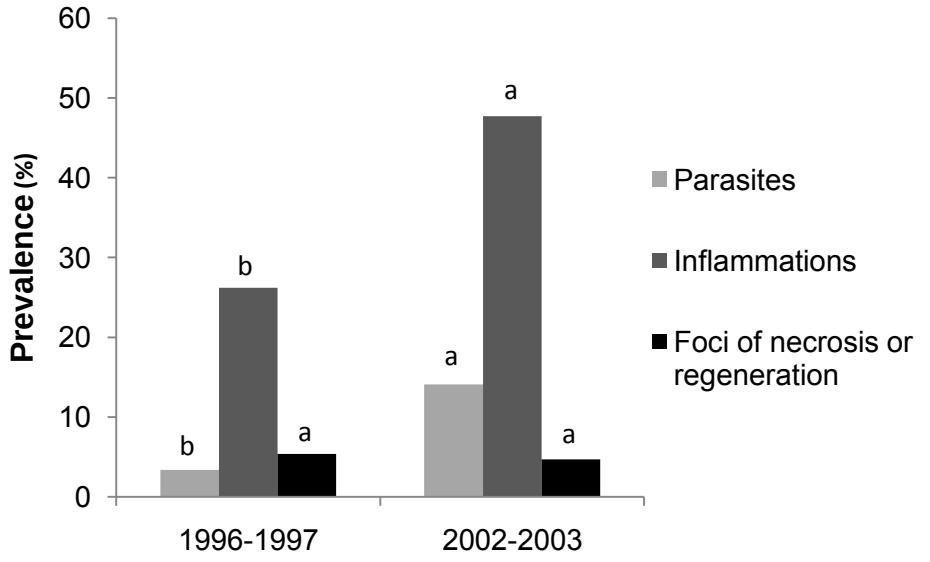


Figure 6

Figure 7

A



B

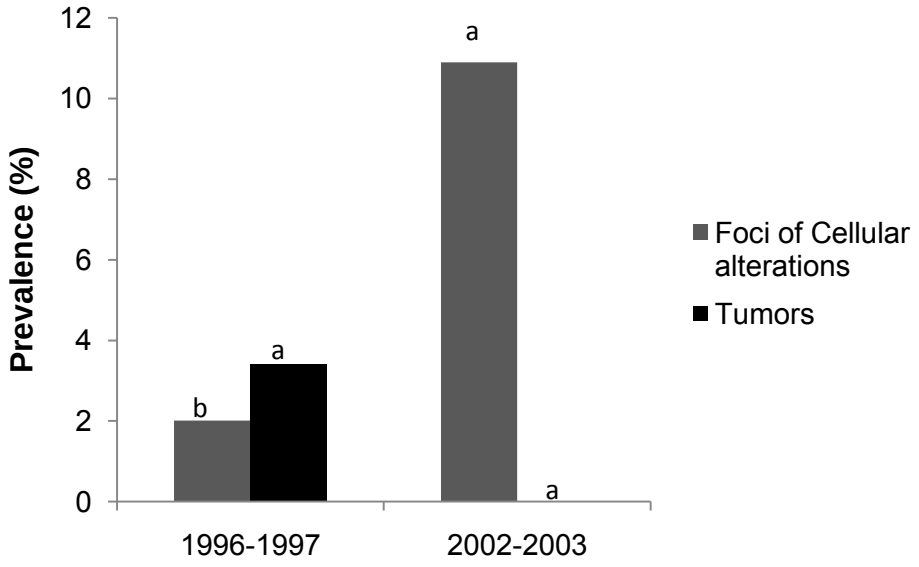


Figure 7

Figure 8

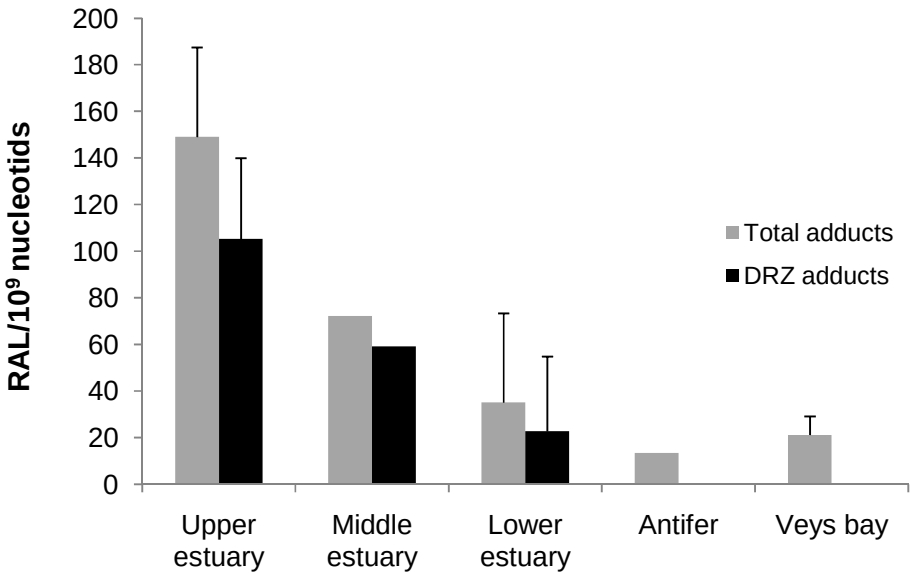
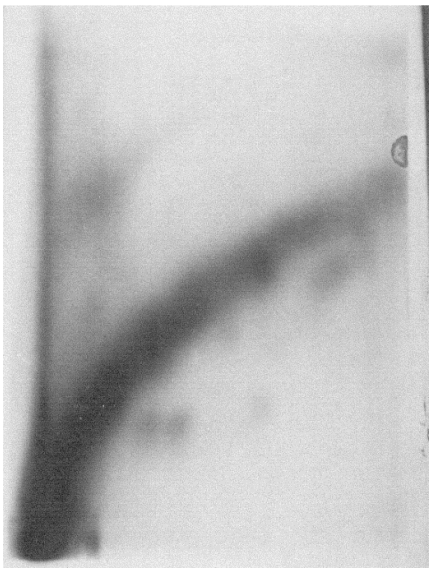


Figure 8

A



B

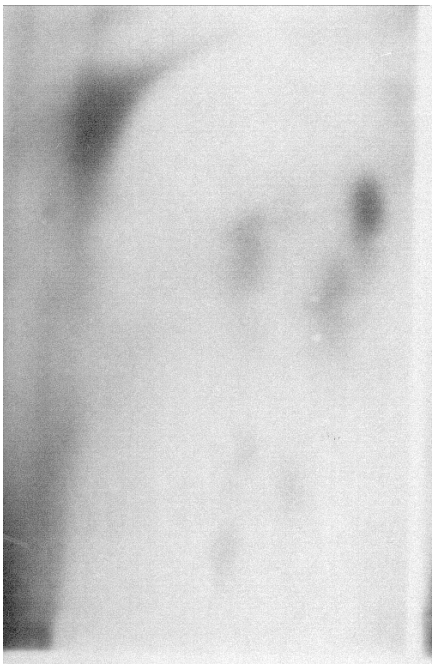


Figure 9

Table 1 : European flounder sampling campaigns in three estuaries from the French Atlantic coast

Sites	Number de fishing campaigns	Period	Number of fish collected	Sex ratio M/F	Total body length (cm)
Seine estuary	9	April 1996	42		
		Sept-Oct 1996	101		
		March 1997	51		
		March 2001	6		
		Sept 2001	10		
		Feb 2002	3		
		June 2002	23		
		Oct-Nov 2002	30		
		Nov 2003	72		
		Total	338	0.71	4.5 - 42.5
Bay of Veys	2	Sept 1996	4		
		April-May 1997	50		
		Total	54	1.41	15.5 - 46
Ster estuary	3	May 2002	28		
		Jan 2003	25		
		May 2004	33		
		Total	86	0.36	19.5 - 49.5

Table 2: Prevalence of liver pathologies in juveniles and adults of European flounder sampled at different locations in the Seine estuary

Sampling sites	Number of fish	Parasites	Inflammations	FNR	FCA	Adenoma	Carcinoma
Upper part	49	18.4 a	26.5 a	10.2 a	14.3 a	0.0 a	0.0 a
Median part	45	2.2 b	26.7 a	4.4 a	4.4 a	0.0 a	0.0 a
Lower part	157	9.5 a	36.9 a	3.2 a	6.4 a	1.9 a	0.0 a
Antifer	87	2.3 b	47.1 a	6.9 a	3.4 a	2.3 a	0.0 a
Seine estuary	338	8.0	36.7	5.3	6.5	1.5	0.0

FCA : foci of cellular alteration, FNR : foci of necrosis or regeneration

Different letters indicate significant differences between sampling sites (Fisher's exact test with Bonferroni's correction)

Table 3

Table 3 : Prevalence of liver pathologies in adults of European flounder (total length > 200mm) from three estuaries from the French Atlantic coast

Sampling area	Number of fish	Parasite	Inflammation	FNR	FCA	Adenoma	Carcinoma
Bay of Veys	46	6.5 b	15.2 b	0.0 a	0.0 a	0.0 a	0.0 a
Ster estuary	85	37.6 a	51.8 a	2.4 a	9.4 a	0.0 a	1.2 a
Seine estuary	243	5.8 b	40.3 a	3.7 a	4.9 a	2.1 a	0.0 a
Median part	***38***	***2.6***	***28.9***	***0.0***	***5.3***	***0.0***	***0.0***
Lower part	***118***	***9.3***	***39.0***	***2.5***	***5.9***	***2.5***	***0.0***
Antifer	***87***	***2.3***	***47.1***	***6.9***	***3.5***	***2.3***	***0.0***

FCA : foci of cellular alteration, FNR : foci of necrosis or regeneration

Different letters indicate significant differences between sampling sites (Fisher's exact test with Bonferroni's correction)

Table 4 : Prevalence of liver diseases in European flounders form different geographical areas of the North-east Atlantic

Sampling area	Year	Number of fish	Parasite	INF	FNR	FCA	Tumor	Reference
Dutch North Sea coast	1985-1989	315	-	11.7	5.4	11.4	0.3	Vethaak and Wester, 1996
Dutch Wadden Sea coast	1988	9608	5.0	-	-	<0.1	<0.1	Vethaak, 1992
Dutch Wadden Sea coast (Den Oever)	1996	240	-	12.5	-	1.7	0	Vethaak et al., 2011
German Wadden Sea coast	1995-2000	1468	-	-	-	19-34	4-38	Koehler, 2004
Baltic Sea	1994	3008	-	-	-	0.6	0.9	Bogovski et al. 1999
Baltic Sea coast	2001-2002	436*	17.7	28.9	-	4.6	0.7	Lang et al., 2006
UK estuaries	2000	204	-	20	-	17.6	1.5	Stentiford et al., 2003
UK estuaries	2006	81	0-6	10-67	5-22	0-28	0-11	Williams et al., 2011
Seine estuary	1996-2003	338	8.0	36.7	5.3	6.5	1.5	This study

INF : inflammation, FCA : Foci of Cellular Alteration, FNR : Foci of Necrosis or Regeneration. (*) Female only