PROGRAMME EUROPEEN INTERREG IVA FRANCE (MANCHE)-ANGLETERRE

FRANCE (CHANNEL)-ENGLAND INTERREG IVA EUROPEAN PROGRAMME

DETERMINATION OF RELEVANT INDICATORS FOR ENVIRONMENTAL MONITORING: A STRATEGY FOR EUROPE (DIESE)

FINAL SCIENTIFIC REPORT

English version

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FOREWORDS

The DIESE project started from existing collaborations between scientists and from their eager to contribute to the improvement of the knowledge and to the management of our common environment. This work was made possible because of the support of many people and institutions. The authors of this report would like to thank these women and men whose support is extremely important. This includes people and institutions from the following groups:

- the European community who launched the call for projects and gave most of the financial support;
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    - the Carpe posienne ;
    - the Réserve Naturelle Nationale de la Baie de Saint-Brieuc ;
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- The people from the Universities and other partners who facilitate the researchers' work.

The authors of this scientific project express their sincere sympathy and gratitude to the people they encounter and work with during the implementation of this program.

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ABSTRACT

The DIESE program (Determination of relevant Indicators for Environmental monitoring: A Strategy for Europe) brought together seven French and British research teams, a private company and the agencies responsible for the management of water bodies of the two countries (ONEMA and the Environmental Agency) in a joint effort to document the ecotoxicological effects related to the presence of chemicals in the environment. To contribute to a better understanding and management of the environment, the program has expanded its efforts to (1) use existing knowledge, or new information acquired during the research program, to identify important biological problems affecting the wildlife, (2) increase our understanding of toxicological mechanisms involved and thus be able to identify the causes of the identified dysfunctions and (3) to hone our expertise and vigilance system in order to better monitor changes in the environment and make appropriate diagnoses.

The first part of the program identified clear biological effects, and using biological tests representative of the mechanisms of action of compounds, identified the responsible compounds present in the environment. In connection with the feminization observed in many fish species in European streams, a search for estrogenic and anti-androgenic compounds was conducted. A new test identifying estrogenic compounds has been developed in roach and the ER-Calux test for anti-androgenic effects has been implemented. The results showed that, in addition to biocides such as triclosan and chlorophène, many aromatic hydrocarbon compounds are likely to disturb the physiology of the living organisms by interacting with the androgen receptor. Six of them were identified in sediment extracts, the benzanthrone, the fluoranthene, the 1,2- benzodiphenylene sulfide, the benzo[a]pyrene, the benz[a] anthracene, and the 9-phenylcarbazole.

The second part of the program aimed at documenting and understanding the mechanisms of action of chemicals leading to physiological changes. This work represents a particular challenge when dealing with molluscs as knowledge about their physiology and endocrinology is still fragmentary. Thus, new technologies including metabolomic and transcriptomic analyses have been implemented in order to obtain a comprehensive picture of the effects on molluscs. Metabolomic research demonstrated that estrogenic compounds are able to alter the metabolism of eicosanoids and amines while transcriptomic strategies identified genes whose expression is altered in intersex clams. Because these genes mainly appear as “male” genes, the result suggests that these profound physiological changes result from demasculinisation of male clams. Proteomic studies have also been carried out to elucidate the mechanisms of action of pollutants on fish physiology. These studies generally included a set of molecular marker measurements in an integrative and ecological perspective. The results showed that not only male fish physiology is altered but also female reproductive status is impaired. Moreover, it appeared that other alterations of the fish endocrine system such as androgenic effects are at work and that the immune system is also subject to chemical pressure including effects from environmental estrogens. Notably, the immune system, like the endocrine system, seems to show periods of particular sensitivity during development. Measurements on growth and on the general metabolism emphasize the importance of environmental conditions in the physiology of aquatic organisms and in particular the inter-site variability due to temperature, hypoxic conditions and fish development strategies. They thus provide a unique perspective that allow to better understand the context and consequences of natural conditions on the population.

In a third part of the program, the research conducted had the objective of developing and testing a biomaker strategy to support the environmental management methodologies. Two lanes of specific studies have been followed. The first was to implement, over all or part of the study area, robust biomarkers to establish a mapping that may highlight the water bodies at risk and provide information on sources of compounds and associated disturbances. The second part of the work aimed at exploring methodologies to take advantage of biomarkers measurements and to integrate them in a

simple and clear index. Partial or comprehensive maps of the Channel area were produced to report the presence of mutagenic or anti- androgenic compounds in the sediments, intersex fish and clams and imposex. These maps may remain to be completed and work will be necessary to confront this information in order to learn relevant lessons for the management of the environment, a goal that the DIESE program has contributed to by providing some necessary and original information.

SUMMARY

I. INTRODUCTION

The health of living organisms, including that of humans, is a delicate balance that can be broken under exceptional situations or that can be destabilized more insidiously under the combined effects of multiple factors that, although less exceptional, are no less harmful. Health is a somewhat general term and difficult to characterize. It includes a set of conditions for the functioning of various organs and physiological functions, including development, growth and reproduction of organisms. So this is a parameter of importance and of particular significance in the case of the many species present in the environment. Our current knowledge allows us to understand the progress of medicine, food safety and personal protection that led to improved longevity and life of people and, therefore, we can foresee that controlling a set of parameters related to the health is essential to the people and the wildlife.

These developments highlight the importance of the scientific knowledge allowing to identify risks, quantify them and to consider remedies. Thus the DIESE program (Determination of relevant Environmental Indicators: A Strategy for Europe) aims both at increasing our knowledge on biological parameters that are essential for the proper functioning of organisms and secondly to identify dysfunctions, to highlight the causes of these problems and to propose tools to make an appropriate diagnosis helping to manage the identified risks.

Considering that some risks are of particular importance, as certain biological and physiological functions are crucial for the proper functioning of organizations, the work conducted during this research program focused (1) on endocrinology, essential for the reproduction, the development and the metabolism, (2) on immunology, a central defence function and (3) on mechanisms of carcinogenesis, a clear example of dysfunctional biological functions. Indeed, data from the literature show that cancers are the leading cause of human mortality in many developed countries, including France (Priou and Barbieri, 2012). Among cancers, the most common is related to the endocrine system, breast cancer in women, prostate cancer in men (Binder-Foucard et al., 2013). Recent work on human reproduction indicate that reproduction is impaired in France for more than 30 years leading to a reduction in the fertility of nearly 1% per year (Rolland et al., 2013) and many endocrine disorders can lead to metabolic dysfunctions responsible at last partly to the second cause of human mortality (Garcia-Mayor et al., 2012). Finally, alterations in the immune system is the fourth leading cause of human mortality (Priou and Barbieri, 2012).

Responsibility for the environment in biological dysfunction is evident. The increase in human cancers cannot be fully explained by the increase in life expectancy and improved screening (Binder-Foucard et al., 2013). Many cancers could thus be avoided. This environmental component is also very obvious when considering alterations of physiological processes in wild organisms as observations of these problems are largely informed and demonstrated experimentally. Various disturbances of the endocrine system are identified and quantified for example in wild aquatic populations and may be reproduced experimentally (Ketata et al, 2008. Minier and Amara, 2008). Wild organisms are good indicators of deleterious effects can occur and good sentinels of biological effects. Thus, this work focused on fish and shellfish living in our environment in order to better document the problems observed and to elucidate the underlying mechanisms.

This report is divided into three parts. The first is dedicated to the identification of obvious biological effects and the associated responsible compounds. The second part allows to document and better understand the mechanisms leading to physiological changes. Finally, the third part reports efforts to develop methodologies that can provide relevant information to better manage our environment. The work illustrates the defined strategy for this program: it is to (1) use the current knowledge to identify environmental problems and biological dysfunctions, (2) increase our

knowledge to better understand and be able to identify the causes of these dysfunctions and (3) improve our expertise system in order to better monitor changes in the environment and make a pertinent diagnosis.

II. IDENTIFICATION OF ACTIVE COMPOUNDS

II.1. Introduction

Living organisms including humans are continuously exposed to mixtures of chemicals in the environment. Nearly 100,000 compounds are considered to be of common use and therefore likely to be part of the mixture in a given environment. Some of these contaminants may have a particularly deleterious activity and there is a legitimate concern about some emerging compounds that are still not measured nor subject to any legislative regulation, including the Water Framework Directive.

The identification of active compounds present in urban sewage and wastewater is thus a critical issue. Regulatory decisions depend on the identification, contamination levels and toxicity. To contribute to the identification of compounds that can be particularly harmful to the health of organizations, the partners of the DIESE program adopted a strategy for identifying compounds in the aquatic environment. This strategy is based on the measurement of the biological activities displayed by the compounds present in environmental extracts. Indeed, rather than inventorying a set of chemical compounds, it appears important to identify those that have a critical effect on key mechanisms. We thus used biological assays to measure key activities that serve as a guide for the identification of chemical compounds.

This report describes the research efforts to identify androgenic and anti-androgenic compounds and to develop a test for the identification of estrogenic and anti-estrogenic compounds in fish.

II.2. Identification of anti-androgens (AA)

Thus far, the identification of EDCs in aquatic environments has been mostly focused on estrogenic compounds, i.e. the steroidal estrogens, and estrogen mimics such as some alkyl phenols. These EDCs act as feminizing agents, however other contaminants act as anti-androgenic (AA) agents which may also inhibit the natural masculinization pathway in aquatic organisms. Surveys of UK WWTP effluents reveal that the majority contain AA as well as estrogenic activity. Reports of AA activity in sediments, water and fish of European rivers have already been described suggesting their presence in the aquatic environment could be widespread (Urbatzka et al., 2007; Weiss et al., 2009; Hill et al., 2010). In addition, the observed feminisation of wild fish (roach, *Rutilus rutilus*) in downstream waters has been correlated to exposure to both anti-androgens and estrogens or to anti-androgens alone indicating that AA compounds maybe affecting the sexual differentiation of wildlife (Jobling et al., 2009). There have also been reports of a high incidence of intersexuality in populations of the clam, *Scrobicularia plana*, in UK estuaries and this condition, termed ovotestis, has been associated with feminisation and/or demasculinisation of the males (Langston et al., 2009 ; Chesman et al., 2006). This finding indicates that EDCs may be present in coastal sediments, and as the nature of estrogenic substances in many sediment sites is already known (Labadie et al., 2007a and 2007b; Peck et al., 2004), then there is a need to determine the prevalence and identify of AA compounds in coastal and estuarine environments.

Anti-androgens can bind to the androgen receptor (AR), but are unable to activate it (AR antagonism). Structures of chemicals containing androgen receptor antagonist properties can be extremely diverse (Rostkowski et al., 2011; Vinggaard et al., 2008) and it is therefore important to use

methods which do not make any assumptions as to the nature of the chemicals involved. These methods include assessment of the extent of AA activity using in vitro androgen receptor screens, and profiling AA activity using an effect-directed analysis approach.

The main aims of the work were to:

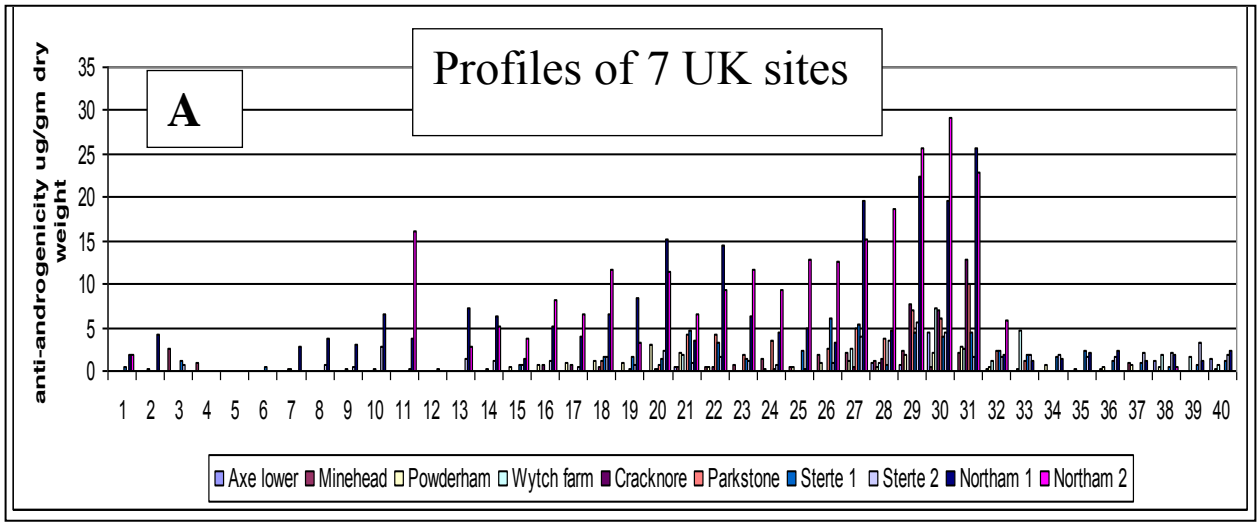
- Investigate AA profiles in coastal sediments of the transmanche Interreg region and determine the identity of the key AA pollutants in contaminated sites.
- Investigate whether sediment –based AA compounds are accumulating in clams sampled from contaminated sites.

Methods were developed to extracts AA activity from sediments and test it using an AR transcription assay (AR-CALUX). Sediments showing high levels of AA activity were fractionated by high performance liquid chromatography (HPLC) and contaminants present in AA active fractions were further analysed by mass spectrometry techniques (GC-EI-MS and LC-ESI-MS). Where available, commercial standards of putatively identified contaminants were tested for AA activity in AR-CALUX and used to confirm structural identity by comparison with retention time and mass spectral data.

Identification of AA active contaminants in coastal sediments

The AA activity present in the sediments was profiled using a combination of chromatography and in vitro assays. The profiles of AA activity were similar at many of the UK sites and most of the AA -active fractions eluted between 11 and 31 mins (Fig. 1A and 1B). The profiles differed from that of the Rouen site which also contained a cluster of non-polar anti-androgenic fractions eluting between 29-39 mins (Fig. 1).

Fractions from the Northam sediment, which was the most contaminated of the sites surveyed, were analysed by MS. The most abundant contaminants present in the AA active fractions were members of the polycyclic aromatic hydrocarbon (PAH) family (Table 1). Some planar structures such as benzo[a]pyrene and benz[a]anthracene showed AA potencies similar to that of the flutamide standard. Other PAH structures with AA activity were heterocyclic molecules containing sulphur or nitrogen, *e.g*. 1,2-benzodiphenylene sulfide and phenylcarbazole. Some structures such as benzanthrone were PAH metabolites. Interestingly the most predominant AA structures that had been previously identified in WWTP effluents were the germicides triclosan and chlorophen (Hill et al, 2010; Rostkowski et al., 2011), but these compounds were present in very low amounts in the samples of coastal sediments. This indicated that the majority of pollution contributing to AA activity in the sediments arose from fossil oil spillages, either from shipping activity or oil refining industries. The identification of PAHs such as benzo[a]pyrene as potential anti-androgens suggest these contaminants may have dual mechanisms of toxicity as planar PAH are normally associated with activation of the aryl hydrocarbon rather than androgen receptor, and as a result are highly carcinogenic.


A
Profiles of 7 UK sites
anti-androgenicity ug/gm dry weight
0
5
10
15
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25
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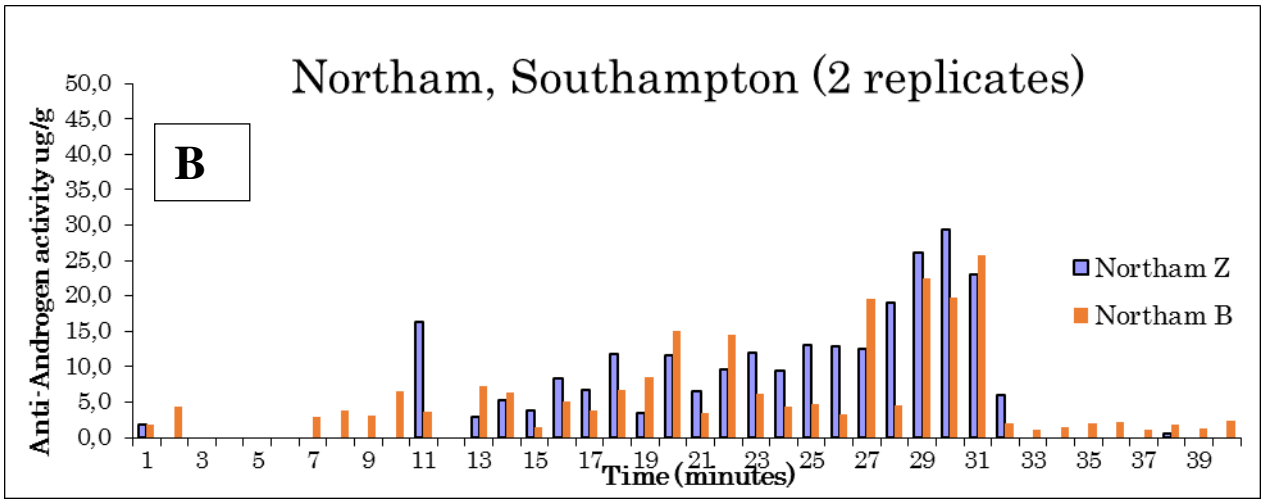
35
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Axe lower
Minehead
Powderham
Wytch farm
Cracknore
Parkstone
Sterte 1
Sterte 2
Northam 1
Northam 2


B
Northam, Southampton (2 replicates)
Anti-Androgen activity ug/g
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10,0
15,0
20,0
25,0
30,0
35,0
40,0
45,0
50,0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
Time (minutes)
Northam Z
Northam B

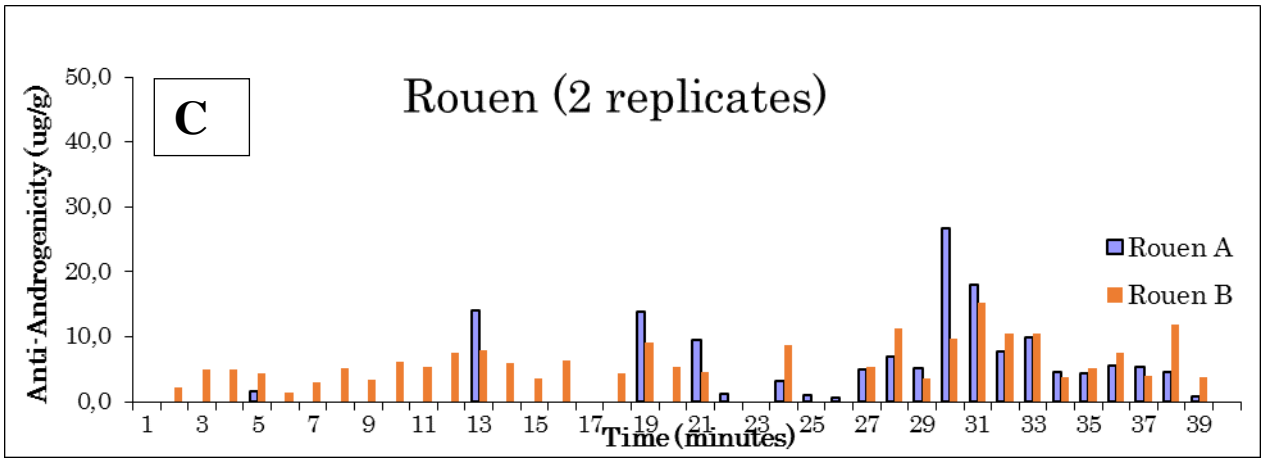
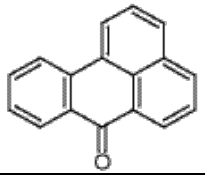
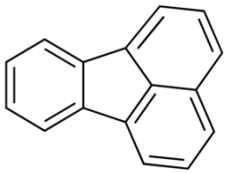
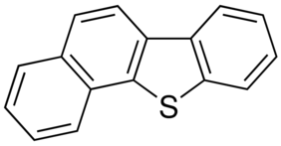
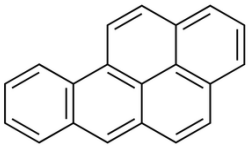
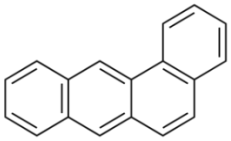
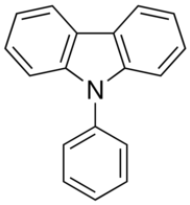


Figure 1: HPLC profiles of AA activity in sediments from A) seven UK sites B) Northam only, C) Rouen only.

Table 1: Identities of some AA contaminants identified in coastal sediments.

Name	Structure	Formulae	CAS No	Potency compared with flutamide
Benzanthrone		$C_{17}H_{10}O$	82-05-3	0.413
Fluoranthrene		$C_{16}H_{10}$	206-44-0	0.955
1,2-benzodiphenylene sulfide		$C_{16}H_{10}S$	239-35-0	0.923
Benzo[a]pyrene		$C_{20}H_{12}$	50-32-8	0.966
Benz[a]anthracene		$C_{18}H_{12}$	56-55-3	1.271
9-phenylcarbazole		$C_{18}H_{13}N$	1150-62-5	0.326

Profiles of AA activity in clams

The question arose as to whether the high levels of AA activity identified in some contaminated sites was bioavailable and able to accumulate in benthic species such as the clam, *Scrobicularia plana*, which has been shown to be susceptible to endocrine disruption (Langston et al., 2007). The levels of AA activity in clams sampled from 4 sites on the Southampton estuary were investigated. Sediments from the Northam and St Denys sites contained 5-20 fold more AA activity then the Woolston and Warsash (Figure 2). AA activity in male clams was significantly higher ($p<0.05$) in Northam and St Denys sites compared with Woolston and Warsash, although this difference was not apparent in the females.

The AA activity present in male and female clams from Northam was profiled using HPLC, and the profiles compared with the extracts from ambient sediment samples (Fig. 3). AA profiles between male and female clams were very similar. Clam profiles reflected those in the sediment, with the exception that three additional non polar fractions eluting at 33, 34 and 38 mins were specific to the clam extracts and not to sediment. Further work is underway to identify the key AA structures accumulating in the clam tissue.

An interesting additional finding is that extracts of male clams contained a highly potent *androgenic* fraction (Fig. 4). However, no androgenic fractions or activities were found in female clams or in the sediment samples. This indicated that the male clams may contain natural androgenic hormones, and further MS analysis of the androgenic fraction revealed it contained dihydrotestosterone (DHT) which is a potent androgenic steroid found in mammals and has also been detected in some molluscs (Janer et al., 2006). This finding needs to be confirmed in further research on clam metabolism, but if definite, then it would indicate that this species could be highly susceptible to androgen receptor antagonists present at many coastal sites in the Interreg cross channel regions.

Further work should enable to determine whether there is a causal relationship between environmental levels of AA activity and intersexuality in *Scrobicularia plana*.

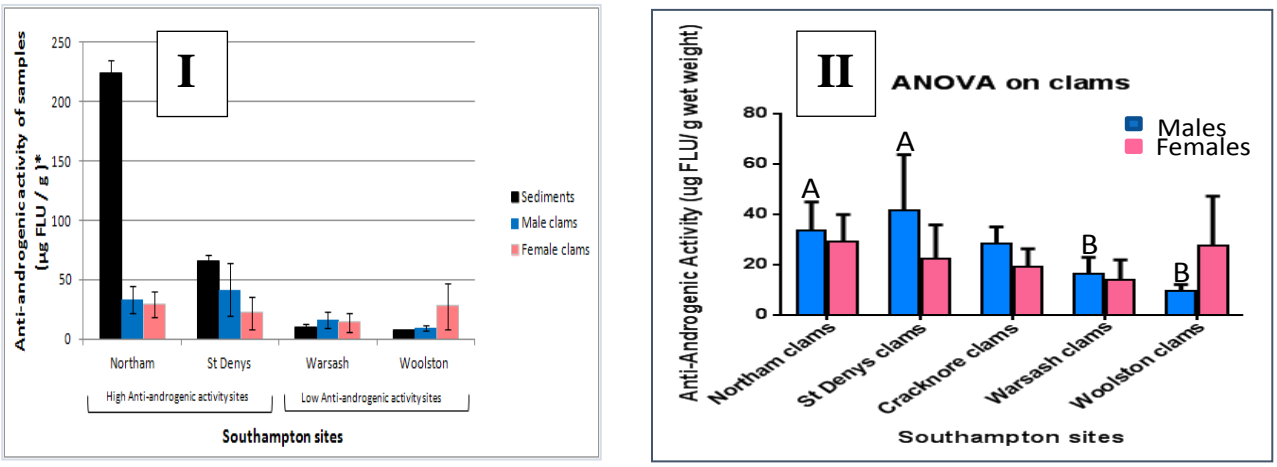


Figure 2. Concentrations of AA activity present tissues of clams sampled from Southampton estuary. AA activity expressed in µg flutamide equivalents/g tissue. Fig I) Concentrations in clams and ambient sediment samples, II) concentrations in clams only, and showing statistical differences in levels of AA activity in male clams (but not females) between sites ($p<0.05$ A compared with B).

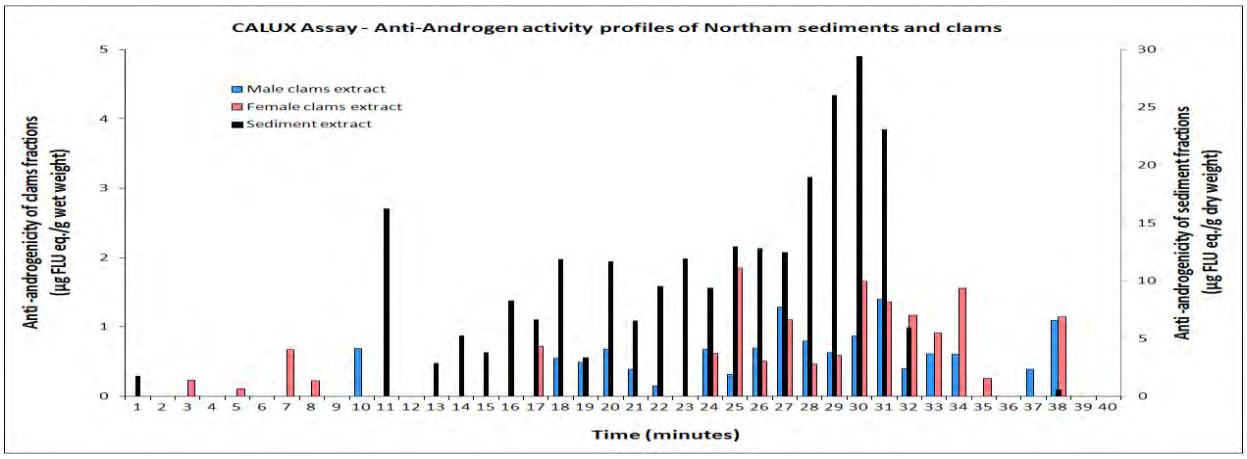


Figure 3. Comparison of HPLC profiles of AA activity in composite samples of either male or female clams or sediments sampled from the Northam site in Southampton estuary.

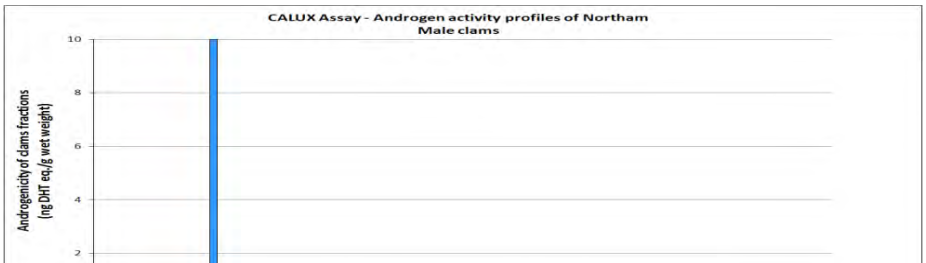


Figure 4. Profile of androgenic activity in a composite sample of male clams collected from Northam site. No androgenic activity was detected in female clams.

II.3. Identification of endocrine disrupting potencies of chemicals using *in vitro* explant cultures

It has been well established that many chemicals released into the environment have the potency to disturb the normal endocrinology of organisms (Sumpter, 1998). Nevertheless, the identification of the responsible molecules and their combined effects on the organisms are complex and still relatively unclear. Accordingly, ecotoxicological tests have been developed to detect and characterize the endocrine disrupting potency of chemicals. As the aquatic medium is the final destination of many contaminants, fish species have been selected for the biomonitoring of environmental health. The roach (*Rutilus rutilus*), a cyprinid fish, is a model of choice to assess endocrine disrupting effects in aquatic systems and especially feminization effects (Minier *et al*., 2000; Tyler, 2007). Among the different biomarkers investigated to study endocrine disruption on fish, vitellogenin (VTG) serves as a reliable biomarker of xenoestrogenic exposure (Nilsen *et al*., 2004; Sumpter and Jobling, 1995 ; Kime *et al*., 1999). VTG is a phosphoglycolipoprotein with a high molecular weight and is a precursor of the vitellin which is a yolk protein constitutive of the eggs. It constitutes the main source of energy during the development of embryos and is thus essential for successful reproduction (Roubal *et al*., 1997). VTG is produced naturally during the oogenesis in adult female liver and transported to the gonads for its incorporation into the eggs. Its synthesis is regulated by endogenous estrogens like 17-β-estradiol (E2) through the link to the estrogen receptors located both in the cytosol and nucleus of the hepatocytes. As VTG can be induced in males by xenoestrogens, VTG induction reflects environmental exposure to estrogenic chemicals.

Recently, scientists have been asked to reduce the use of vertebrates for ethical reasons and to develop ecotoxicological tests. In line with these requirements, alternative methods including *in vitro* bioassays have been suggested to be developed (Walker *et al*., 1998). *In vitro* tests cannot replace *in vivo* studies on whole animals, but they can increase our knowledge and especially our understanding of the mechanisms of action of EDCs. The advantages of *in vitro* tests are their reproducibility, sensitivity and cost effectiveness compared to *in vivo* methods. It has been put forward that the measurement of VTG induction in the medium of cultured fish hepatocytes should be used as an identification test of chemicals with estrogenic activity (Pelissero *et al*., 1993; Smeets *et al*., 1999a). By maintaining the structure of the liver, the explant cultures are closer and more representative of the *in vivo* conditions than hepatocyte cultures (Hurter *et al*., 2002). Thus, they could be used to assess the estrogenic or antiestrogenic potency of both the parental compound and their metabolites (Navas and Segner, 2006). Moreover, this new technique will simplify the protocol used for hepatocyte cultures because the isolation of the cells is not needed.

The aim of this study was therefore to develop a new *in vitro* bioassay to evaluate the potential estrogenic and anti-estrogenic activity of environmental chemicals by measurement of the VTG synthesis. The use, for the first time, of roach liver explants may help document the observed effects *in situ* on this particular species (Minier *et al*., 2000; Maltret-Geraudie *et al*., 2008).

Setting up of the liver explant culture

Adult roach were collected and anaesthetized. The liver was removed in sterile conditions before being perfused with a rinsing solution to eliminate blood. Pieces of about 3 mm^3 (5-20 mg) were cut from the liver, constituting the explants, which were then placed in 24 well plates with one explant per well. 1 ml of culture medium without phenol red and completed with 1% glutamine and 1% antibiotics (penicillin, streptomycin and amphotericin B) was added to each well. Plates were then incubated at 25°C and held on an orbital shaker (40 rpm) for up to 120 hours, ready for testing chemicals. The cell viability was assessed by the measurement of non-specific esterase activity using a fluorescein diacetate (FDA) hydrolysis assay (Larsson and Nygren, 1989; Tutundjian *et al*., 2002) and the estrogenic response was assessed by measurements of vitellogenin (VTG) in the culture medium using a sandwich homologous carp enzyme-linked immunoassay. Cell viability tests showed that viability of the explants incubated in medium culture did not significantly decrease up to 72 hours but was then significantly altered at 96 hours.

Estrogenic activity of environmental chemicals

A clear dose-effect relationship could be obtained under the stimulation of the VTG production using E2 for either 48, 72 or 96 hour incubation periods (Fig. 5). VTG synthesis was only detectable (0.2 $ng.mL^{-1}$) for E2 concentrations above 1 nM. Moreover, VTG levels after E2 exposure to 100 and 1000 nM were significantly higher (6.9 and 21 $ng.mL^{-1}$ respectively) than those resulting from lower E2 doses (0-10 nM). Several known environmental pollutants were used to study their relative estrogenic potential using roach liver explants. No VTG dose-dependent response for BPA, BP and NP could be established because of the cytotoxicity of the compounds (Table 2). After 48 hour exposure to 500 µM bisphenol A (BPA), butylparaben (BP) or 4-nonylphenol (NP), FDA hydrolysis did not exceed 0.49 $FU.min^{-1}.mg^{-1}$ (± 0.17) which is significantly lower than the control. There was no measurable VTG induction after dosing with 10 µM of any compounds and significant responses were only obtained with 100 µM (Fig. 6). Among the compounds tested, NP had the lowest estrogenic potential with VTG induction reaching only 18.8 % of the control (100 nM E2). The estrogenic responses of 100 µM BPA or BP were significantly higher than NP. EE2 was the most efficient compound to induce VTG with a significant VTG response measured from 1 nM (Fig. 7). The calculated relative potency of the tested compounds is summarized in table 3.

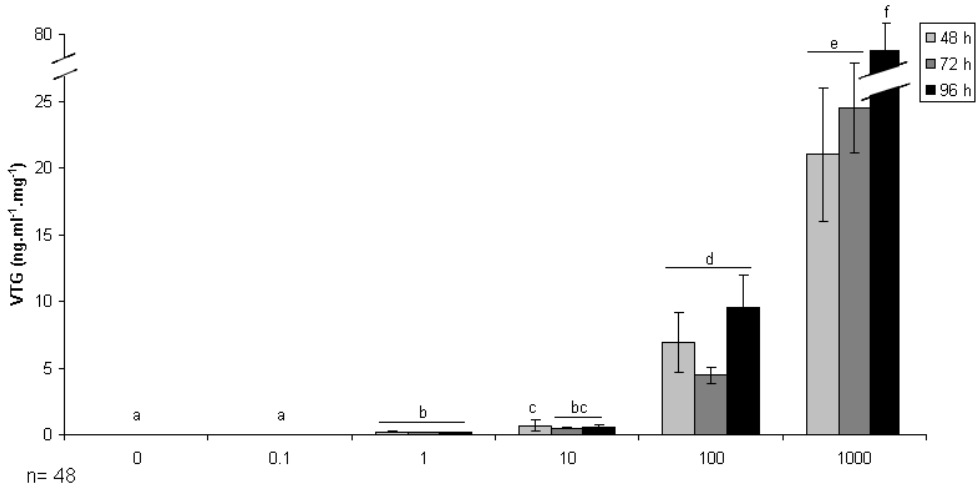


Figure 5: VTG concentration in culture media of roach liver explants treated with different concentrations of E2 (17-β-estradiol) for 48, 72h and 96h. Results are given as mean ± C.I. Values with different letters indicate statistically significant differences between the groups ($p< 0.05$).

Table 2: Explant viability using FDA hydrolysis activity ($FU.min^{-1}.mg^{-1}$) after exposure to different compounds. BPA: Bisphenol A, BP: butylparaben, NP: 4-nonylphenol. Results are given as mean ± C.I. Values with different letters indicate statistically significant differences between the groups ($p< 0.05$).

Compound exposure concentration	control	BP	BPA	NP
10 µM	1.79 (± 0.34)^{a}	0.96 (± 0.09)^{b}	1.13 (± 0.50)^{ab}	1.43 (± 0.83)^{ab}
100 µM	1.79 (± 0.34)^{a}	1 (± 0.01)^{b}	1.1 (± 0.50)^{ab}	1.2 (± 0.51)^{ab}
500 µM	1.79 (± 0.34)^{a}	0.05 (± 0.04)^{c}	0.06 (± 0.003)^{c}	0,49 (± 0.17)^{bc}

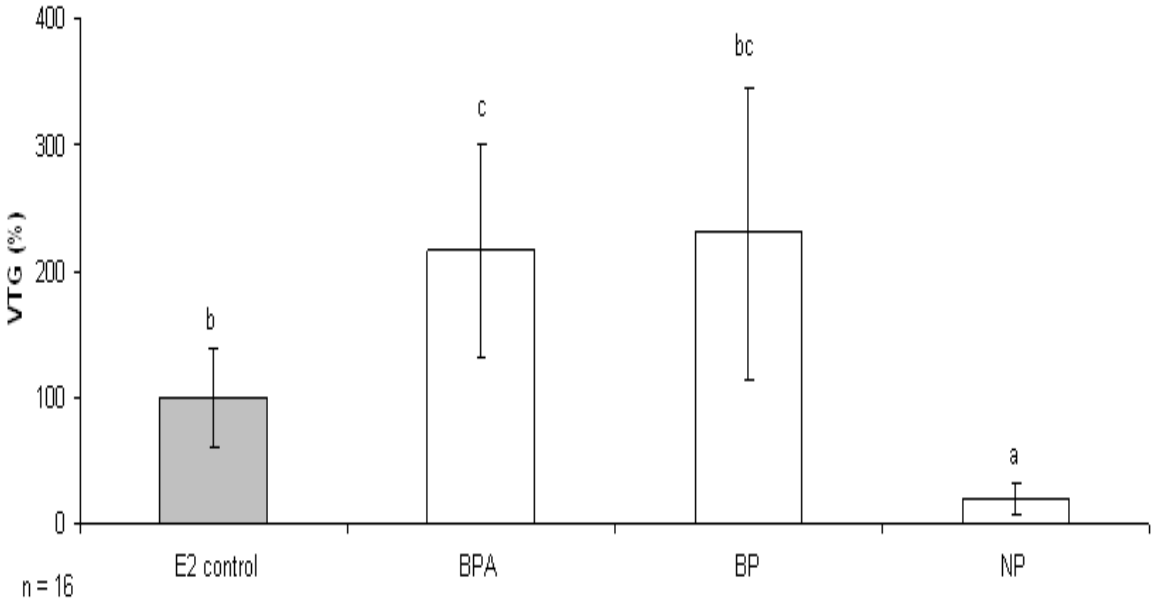


Figure 6: VTG concentration in culture media of roach liver explants treated with 100 nM E2 or 100 µM bisphenol A (BPA), 100 µM butylparaben (BP) and 100 µM 4-nonylphenol (NP) for 48 h at 20°C. VTG concentration is expressed as percentage of the E2 control. Results are given as mean ± C.I. Values with different letters indicate statistically significant differences between the groups ($p< 0.05$).

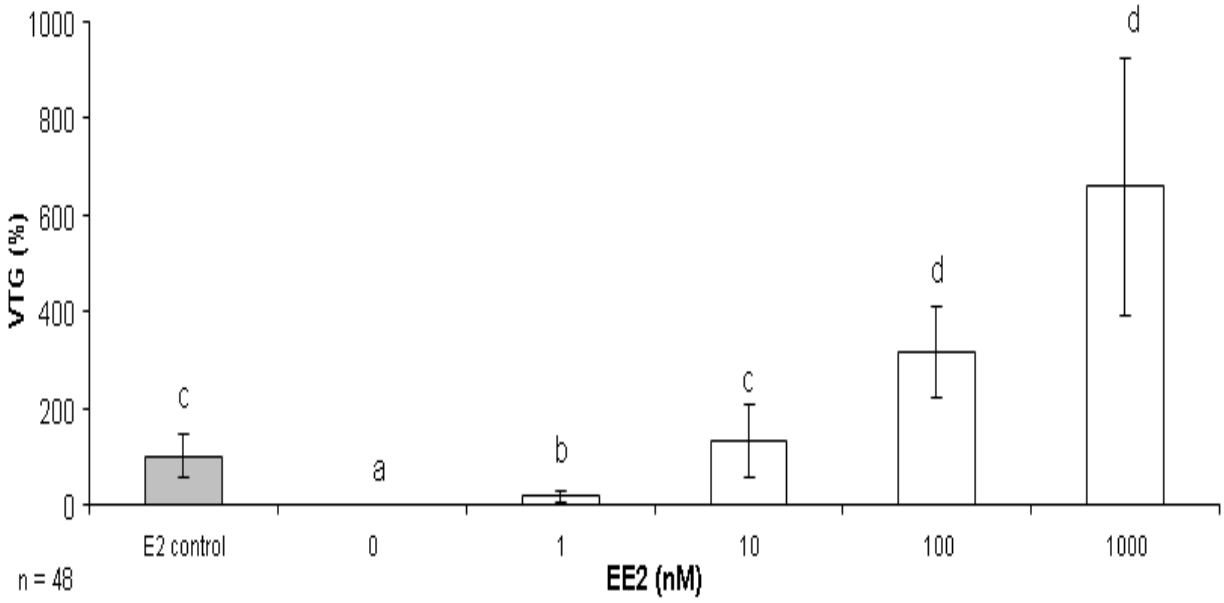


Figure 7: VTG induction after exposure to EE2 (17-α-ethinylestradiol) for 48 h at 20°C. VTG concentration is expressed as percentage of the E2 control. Results are given as mean ± C.I. Values with different letters indicate statistically significant differences between the groups ($p < 0.05$).

Table 3: Estrogenic potency of E2 (17-β-estradiol), EE2 (17-α-ethinylestradiol), BPA (bisphenol A), BP (butylparaben) and NP (4-nonylphenol) calculated from VTG concentration measurements in culture media of roach liver explants

Compound	**Estrogenic potential**
E2	1
EE2	10
BPA	0,01
BP	0,01
NP	0,0002

Anti-estrogenic activity

In order to verify that the test could also be used to assess anti-estrogenic activity, liver explants were incubated simultaneously with E2 and tamoxifen, a known anti-estrogenic compound in breast mammal cells (Cameron *et al*., 2000). The addition of 0.1 µM tamoxifen associated with 100 nM E2 to the explant culture was responsible for a significant decrease of the VTG response compared to an exposure to E2 alone (Fig. 8). 10 µM tamoxifen totally inhibited the estrogenic effect of E2 under the test conditions.

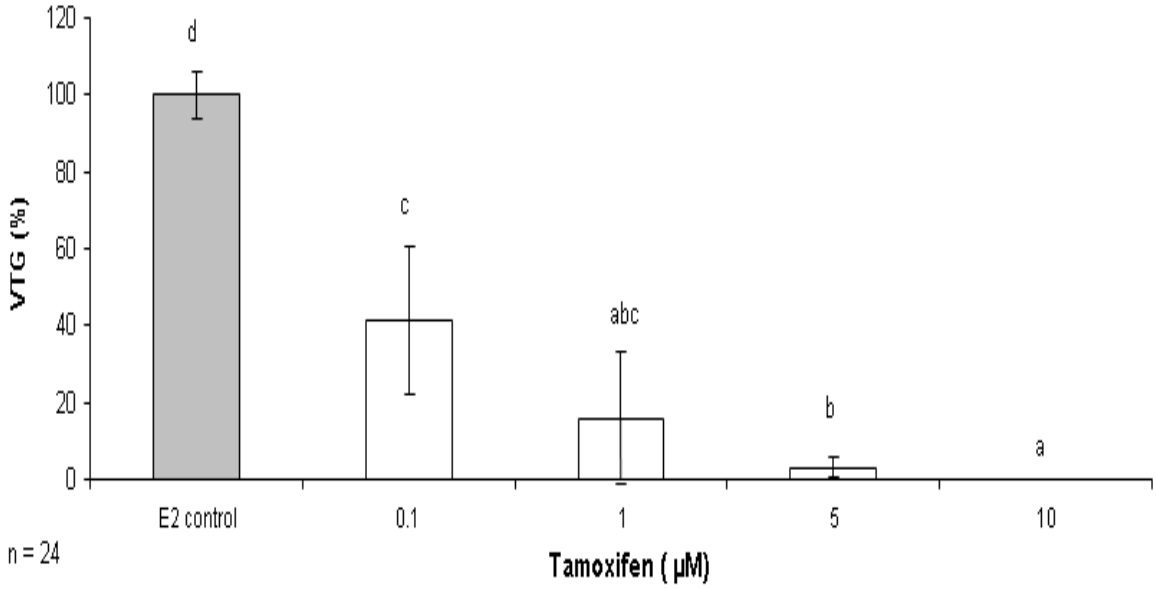


Figure 8: VTG concentration in culture media of roach liver explants treated with different concentrations of tamoxifen associated with 100 nM E2 for 48 h at 20°C. VTG concentration is expressed as percentage of the concentration obtained using 100 nM E2. Results are given as mean ± C.I. Values with different letters indicate statistically significant differences between the groups ($p < 0.05$).

Conclusion

The roach liver explant culture is a sensitive assay for the ecotoxicological evaluation of the estrogenic or anti-estrogenic potency of chemicals. Liver explants provide a realistic *in vitro* model of the entire organ as the different cell types are included and the cell to cell interactions are maintained. This test shows interesting potentials and could be associated with other assays to complete our knowledge on estrogenicity of environmental compounds, metabolites or mixtures.

III. IDENTIFICATION OF MECHANISMS OF TOXICITY

III.1. Introduction

It is very difficult to demonstrate the involvement of any particular chemical in a biological dysfunction in wild organisms sampled in the environment. Indeed, many factors can contribute to an observed situation and any compound is then only a contributor for which it is difficult to perceive the degree of involvement. However, with increasing knowledge and understanding of the interactions of the compound with the biological components of an organisms, the plausibility of the involvement of the compound can be highlighted and a "mode of action" can be discovered. This mechanism is important because it allows to measures the alterations that the compound may be responsible for and thus trace its possible involvement.

To identify the mechanisms by which a compound can alter a biological function, it is essential to understand how this function is regulated under “normal” conditions. This knowledge is often incomplete and it is necessary to explore and elucidate the endocrine and immune systems of living organisms. This is especially true for invertebrate species including the molluscs species studied in this project. The endocrinology of molluscs is unfortunately poorly understood and is substantially different from the much better known mechanisms of the vertebrates (Ketata et al., 2008). However many gray areas may also be present in the physiology of vertebrates, particularly in some species living in European water bodies that are not "model organisms" and have yet been chosen in this program because they can provide information about the environment in which they live, that is to say, our environment.

This chapter is dedicated to the work that helped deepen both our understanding of important physiological mechanisms (especially in relation with the endocrine and immune systems) and the mechanism of action of pollutants.

Two mollusc species have received particular attention within this DIESE program, the blue mussel, *Mytilus edulis*, and the clam, *Scrobicularia plana*. The first is a bivalve extensively studied in the context of environmental studies and is present in the whole coastline of the eligible areas of the project. The second is a bivalve well distributed in the estuaries of the study area. It lives buried in the sediment and is impaired with alterations of sexual differentiation during gametogenesis leading to the presence of both male and female cells within a single gonad and thus characterizing the phenomenon of intersex (Langston et al. 2007).

Five fish species living in the eligible study area received particular attention in this DIESE research program : the sea bass, *Dicentrarchus labrax,* and the flounder, *Platichthys flesus,* are representative of the marine area; the roach, *Rutilus rutilus*, the bullhead, *Cotius cotius*, and the three spine stickleback, *Gastrorosteus aculeatus,* are representative of the freshwater area. Except for the bullhead, these species have been extensively studied and the conducted work has been performed on solid scientific grounds. The choice of the bullhead has been made in order to study special aspects of endocrine disruption, i.e. androgenic effects.

Two main research axes have been developed in this program: the immune system and the endocrine system. These were further studied in an integrated assessment of flounder health status.

III.2. Identification of mechanisms using a metabolomic and genomic approach in the blue mussel *Mytilus edulis*

III.2.1. Introduction

Estrogenic contaminants in the aquatic environment are associated with feminisation of male fish, however their effects on some invertebrate species, such as bivalve molluscs, have yet to be characterised. The lack of knowledge is an obvious obstacle to the identification of effects driven by pollutants as, without knowing the endocrine mechanisms involved, we cannot measure nor observe the associated disturbances. To increase our knowledge and to discover relevant biological indices, a metabolomic approach have been conducted. Indeed, the study of the metabolome, *i.e.* all metabolites of an individual allows us to track a large number of parameters simultaneously. In the absence of knowledge, monitoring thousands of parameters can potentially allow the identification of those that are showing changes in a particular condition of interest.

This approach has been implemented on samples of mussels exposed to a range of doses of the natural estrogen, 17-β estradiol (E2). Approximately 10,000 signals were recorded using mass spectrometry for each sample and allow differentiating the exposure conditions (Fig. 9). Among the discriminating signals, some have been identified and used to unravel potential mechanisms of action of steroids in molluscs. This was the case of prostaglandins and amines that were differentially expressed in the presence and absence of estradiol and led to further examine mechanisms linked to serotonin (5-HT) and prostaglandins (such as the COX enzymes).

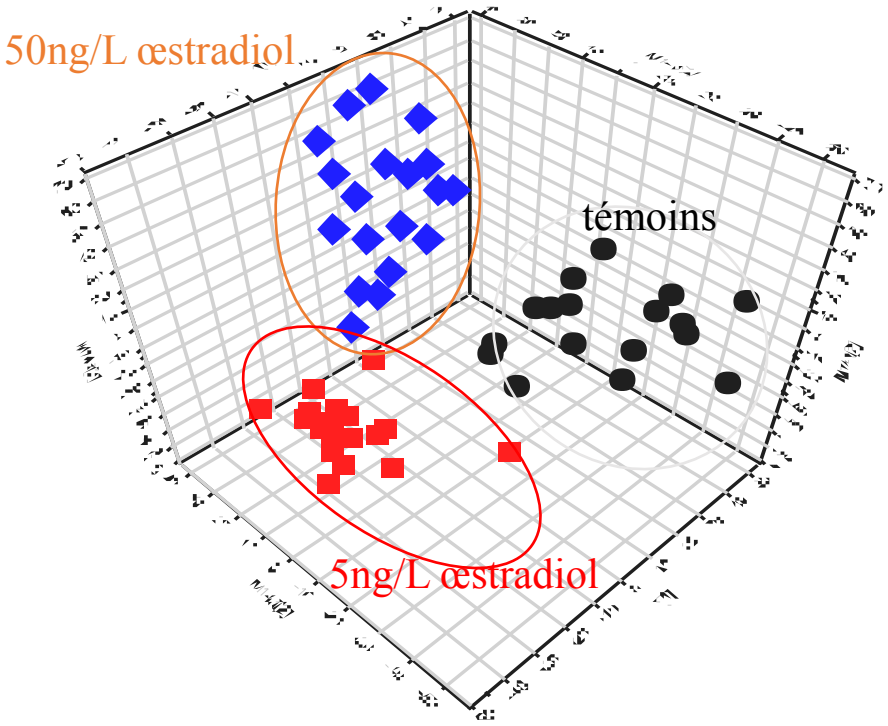


Figure 9 : PLS-DA scores 3D plots of fraction from female and male gonad extracts performed from mussel exposed to (●) 0; 5 (■) and 50 (♦) ng E2/L.

III.2.2. Identification and characterisation of 5HT, COX 1 and CYP3A genes in Mytilus edulis

Gametogenesis is a crucial step in the process of reproduction and is subject to hormonal control from serotonin (5-HT), prostaglandins (synthesized by cyclooxygenase, COX) and steroids such as 17β-estradiol (E2). We examined the responses of 5-HT receptor and the expression of COX gene in mussels, *M. edulis*, exposed to estrogenic compounds during different stages of their reproductive cycle.

The monoamine serotonin (5-HT) is an important mediator of reproduction. Administrations of 5-HT have been shown to induce spawning in bivalves (Matsutani and Nomura, 1982; Gibbons and Castagna, 1984; Matsutani and Nomura, 1987; Ram et al., 1993). In addition, levels of 5-HT have been shown to decrease after spawning in the gonads of the hermaphroditic scallop *Argopecten purpuratus* (Martinez and Rivera, 1994; Martinez et al., 1996) supporting the hypothesis of an involvement of monoaminergic mechanisms during spawning processes. Some studies have indicated that estrogens potentiate 5-HT-induced spawning (Osada et al., 1992; Wang and Croll, 2006) which may be achieved through an induction of the formation of 5-HT receptor (Osada et al., 1998). 5-HT is also implicated in sexual differentiation. During the sexual differentiation stage, higher levels of E2 and lower levels of 5-HT content are found in female brain tissues of the fish *Oreochromis mossambicus* compared to the levels found in male brains (Tsai et al., 2000). This inverse relationship of 5-HT and E2 has also been described in the scallop *A. purpuratus* (Martinez and Rivera, 1994).

The aim of this study was to examine changes in the relative gene expression of a *COX* and a *5-HT receptor* gene in the marine mussel, *M. edulis* exposed to estrogens. These changes were analyzed first in mature mussels exposed to E2 and, in a second experiment where the majority of the individuals were at early gametogenesis stages of the reproductive cycle, in mussels exposed to E2, and the xeno-estrogens EE2 and estradiol benzoate (EB).

The results show that environmentally-relevant levels of the natural estrogen, E2, as well as the synthetic estrogen, EE2, induce alterations, dependent on reproductive stage, in the expression levels of *5-HT receptor* and/or *COX* in the marine bivalve *M. edulis*. The expression levels of the 5-HT receptor and COX enzymes mRNA were different in males and females, indicating a specific role in different gender. In addition, the results showed that environmentally relevant concentrations of the natural estrogen, E2, and the synthetic estrogen, EE2, induce changes in the expression levels of 5-HT and / or COX in mussels. In particular E2 decreases the level of expression of COX enzymes in females while the steroid has no effect on males (Fig. 10). On the expression of 5-HT receptor, exposure to E2 resulted in a decreased expression of the gene in mature males and females. In contrast, exposition during early stages of gametogenesis generated a very sharp increase in the receptor expression (Fig. 11).

These results indicate that important endocrine parameters can be altered by the exposure to estrogens and thus allow to hypothesise on the mechanism of disturbances observed in the wild that may be linked to estrogen exposure (and Blaise al, 2003. Gauthier-Clerc et al, 2006). We could also show that the effect differ depending on the stage of sexual development. This is in line with recent discoveries on effects on the endocrine system and highlight the difficulty of understanding the risks associated with compounds interacting with the endocrine or the immune system as the effects do not depend only on dose but also time of exposure.

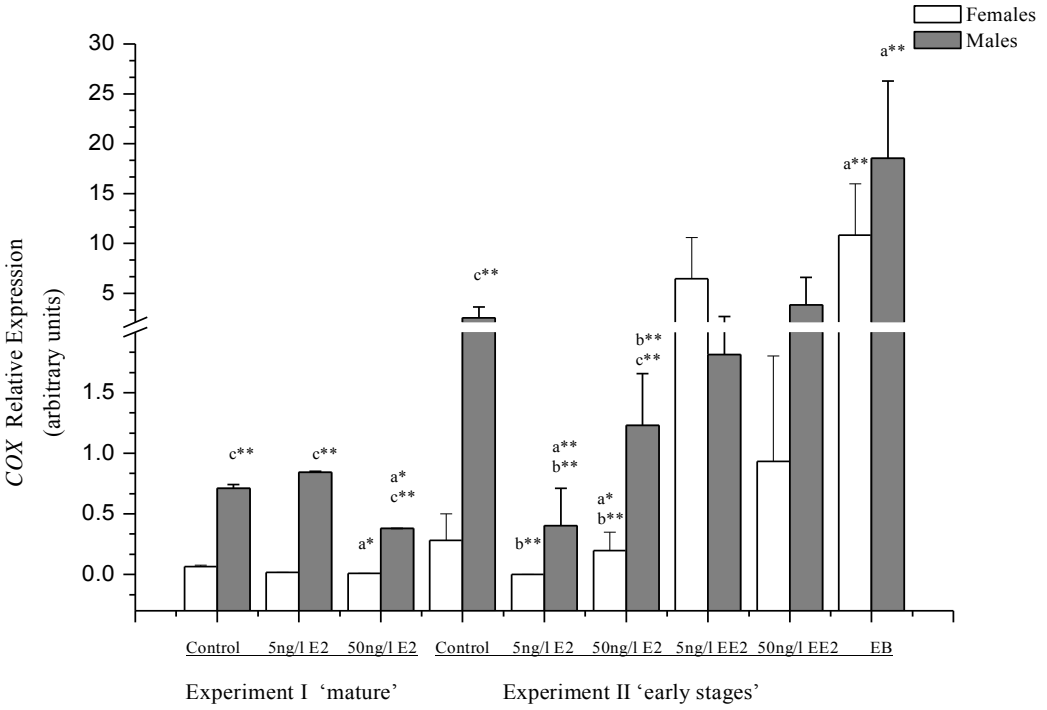


Figure 10. *COX* gene expression of female and male mussels exposed to 5 ng/l and 50 ng/l of E2 and EE2 and 200 ng/l of E2-B during early and late maturation stages. Mean data are plotted ± SEM. ** Indicates significant differences of $p<0.01$ from groups with same letter, * indicates $p<0.05$..

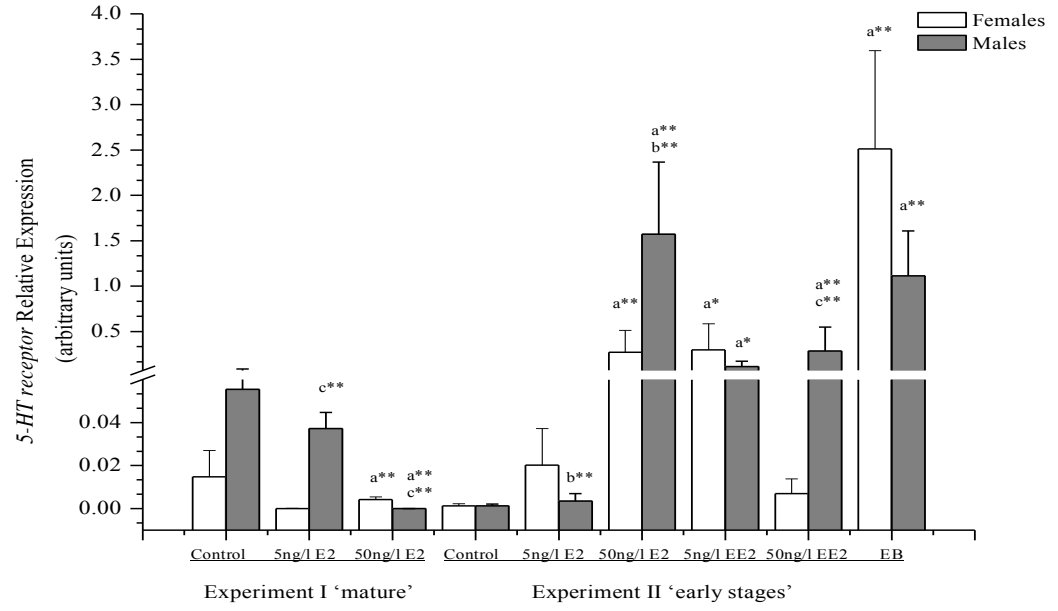


Figure 11: *5-HT* gene expression of female and male mussels exposed to 5 ng/l and 50 ng/l of E2 and EE2 and 200 ng/l of E2-B during early and late maturation stages. Mean data are plotted ± SEM. ** Indicates significant differences of $p<0.01$ from groups with same letter, * indicates $p<0.05$.

Follow-up investigations concentrated on the steroid metabolising pathway. The cytochrome P450s (CYPs) comprise a superfamily of heme-containing proteins involved in the oxidative metabolism (phase I biotransformation) of endogenous and xenobiotic compounds including steroids and environmental pollutants. P450 enzymes in the *CYP3A* subfamily catalyze the hydroxylation of testosterone at the 6β position (Arukwe and Goksøyr, 1997) and metabolize a wide range of chemically diverse lipophilic organic compounds such as environmental pollutants, drugs and steroids (reviewed by Maurel, 1996).

CYP3A-like proteins have been characterized mainly in mammals and other vertebrate species including fish, (reviewed by Schlenk et al., 2008). In invertebrates, *CYP3* gene homologues have been described in the tunicate *Ciona* spp. (Verslycke et al., 2006) and, most recently, in mussel *Mytilus californianus* (Zanette et al., 2010). Cross-reactivity with antibodies specific for vertebrates also suggests that CYP3A-like proteins are present in the digestive gland of *M. edulis* and *M. galloprovinciallis*. (Peters et al., 1998; Shaw et al., 2004).

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Here we describe the isolation of two *CYP3A*-like genes from mussel (*M. edulis*) gonad tissue (GenBank AB479539 and GenBank AB479540). In order to identify if environmental pollutants are potential targets for *CYP3A-like* gene, mRNA expression was investigated in gonads of mature mussels exposed to 17β-estradiol (E2), tributyltin (TBT) and a 10% sewage treatment works (STW) effluent extract. Chemical analysis of free and total estradiol and tin content were conducted in parallel to confirm uptake. In addition, sexual dimorphism and natural variation of *CYP3A-like isoform* expression was investigated in individuals at different periods of their reproductive cycle (Fig. 12 and 13).

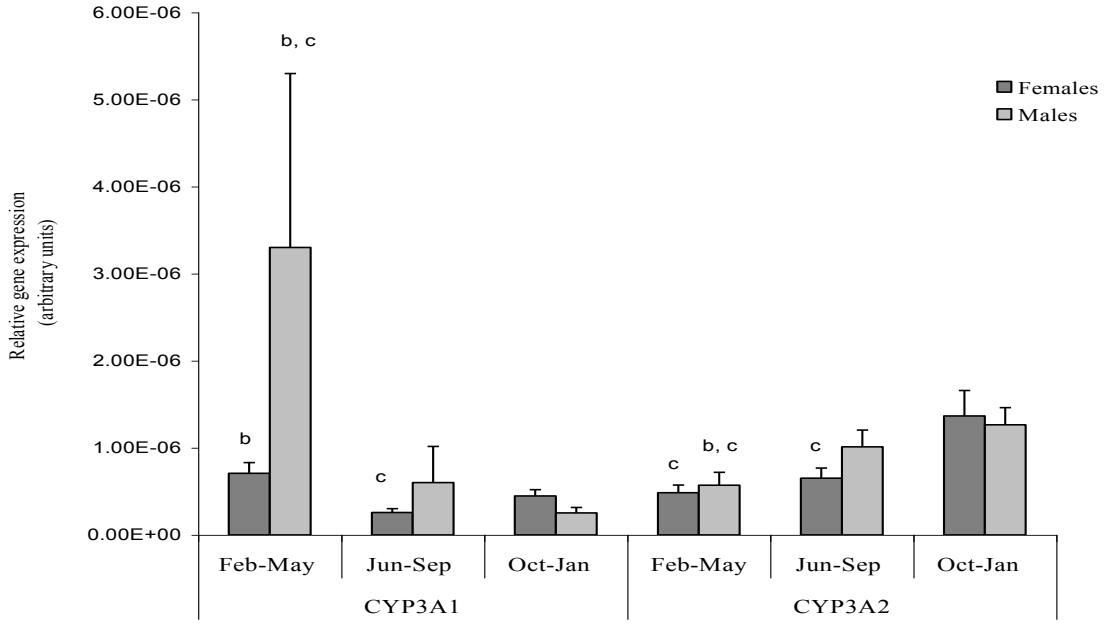


Figure 12: Quantified levels of both *CYP3A* genes in seasonal samples. "b" represents a statistically significant difference ($p<0.05$) compared with the corresponding value for the same sex at Jun-Sep period; "b" represents a statistically significant difference ($p<0.05$) compared with the corresponding value for the same sex at Oct-Jan period.

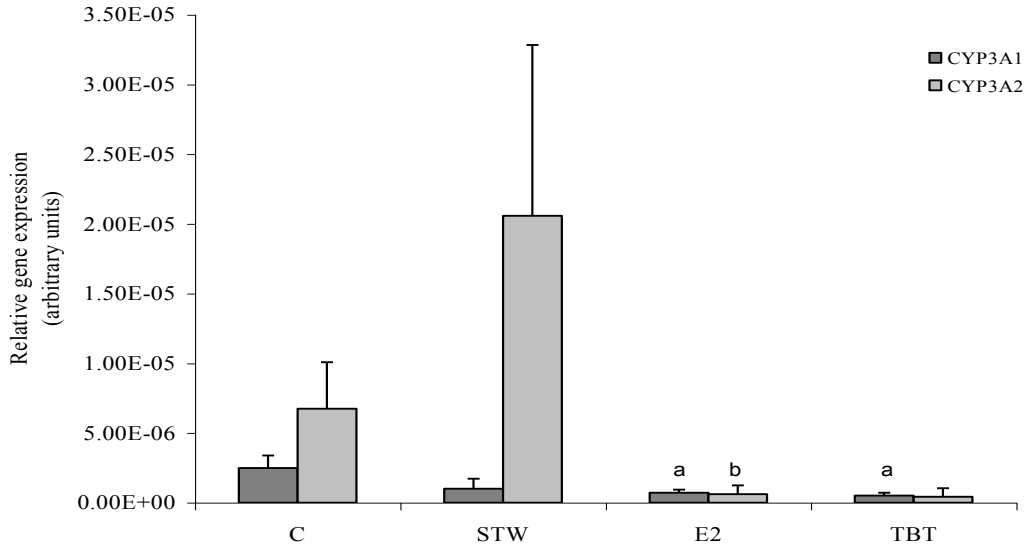


Figure 13: Quantified levels of both *CYP3A* genes in E2 (200 ng/l), TBT (100 ng Sn/l) and 10% STW (previously shown to be estrogenic to fish, Tyler et al.) exposed *M. edulis* using quantitative real-time PCR. "a" represents a statistically significant difference ($p<0.05$) compared with the corresponding control group; "b" represents a statistically significant difference compared with the corresponding value for the STW regime. C, control group; STW, sewage effluent treatment group; E2, E2 treatment group; TBT, TBT treatment group.

In conclusion, two mussel *CYP3A-like* genes were isolated and their mRNA expression profiles of natural variation and following exposure to selected endocrine disruptors studied. Different patterns in natural variation of *CYP3A-like isoforms* suggest that they may play different roles in the physiology of mussels. Expression of *CYP3A-like isoform1* mRNA in mussel gonads was modulated by E2 and TBT. *CYP3A-like isoform2* mRNA levels were also modulated by, as yet, unidentified organic compounds present in a 10% STW extract.

III.2.3. Investigation of any potential disruption of the reproductive cycle in Mytilus edulis following exposure to estrogenic compounds

Treated and untreated municipal and industrial effluent, agricultural practices and livestock wastes are principal sources of hormonal contamination in aquatic environments (Matozzo et al., 2008). E2 is one of the most potent and biologically active estrogen synthesised in female ovaries, while synthetic derivatives such as ethinyl-estradiol (EE2) and estradiol benzoate (EB) are used in formulation of contraceptives, treating menopausal disorders or in controlled reproduction of domestic livestock respectively. E2 concentrations near sewage treatment works have been reported to range from 2.7 ng/l to almost 50 ng/l in UK Rivers. Whilst investigations (in field and under laboratory conditions) have been conducted to assess E2 and EE2 effects on bivalves (Canesi et al., 2007; Canesi et al., 2008; Wessel et al., 2007; Quinn et al., 2004), less is known about EB and its interference with the endocrine system, especially in marine organisms. EB is an estradiol analog, which contains a benzyl ester at the C-3 position. EB is commonly used to supply estrogen for female livestock, stimulate oestrus and ovulation and also for prevention and treatment of many postpartum diseases. Relevantly Mori (1969) reported an acceleration of sexual maturation and incidence intersex in *C. gigas* injected for 10-14 days with EB.

Previously, we reported no changes in *VTG* and *ER1 or ER2* mRNA expression in mussels exposed to E2 at the ripe stage of their gonadal maturation cycle (Puinean et al., 2006, Interreg II-III). Building on this observation, the aim of this study was to determine if it was possible to experimentally induce *VTG* and/or *ER2* mRNA expression in gonad tissue by exposing mussels, *M. edulis,* at different times of their reproductive cycle while using E2, as well as other synthetic estrogens EE2 and EB.

We have reported results from two exposure experiments carried out while mussels were at their maturation stage, in April 2006 and April 2007 and a further exposure experiment carried out while mussels were at their early stage of gametogenesis, in February 2008. Both experiments conducted using mature mussels failed to record statistically significant differences in *VTG* mRNA expression levels after 10 days exposure to E2 (5, 50 and 200 ng/l) (Fig. 14 and 15 'mature'). In contrast, mussels at an early stage of gonadal development do show statistically different *VTG* mRNA expression levels following E2 exposure that varies according to dose (Fig. 14 and 15 'early gametogenesis'). These results suggest a possible regulatory mechanism for estrogen signalling, which varies throughout the reproductive cycle (Zhu et al., 2003).

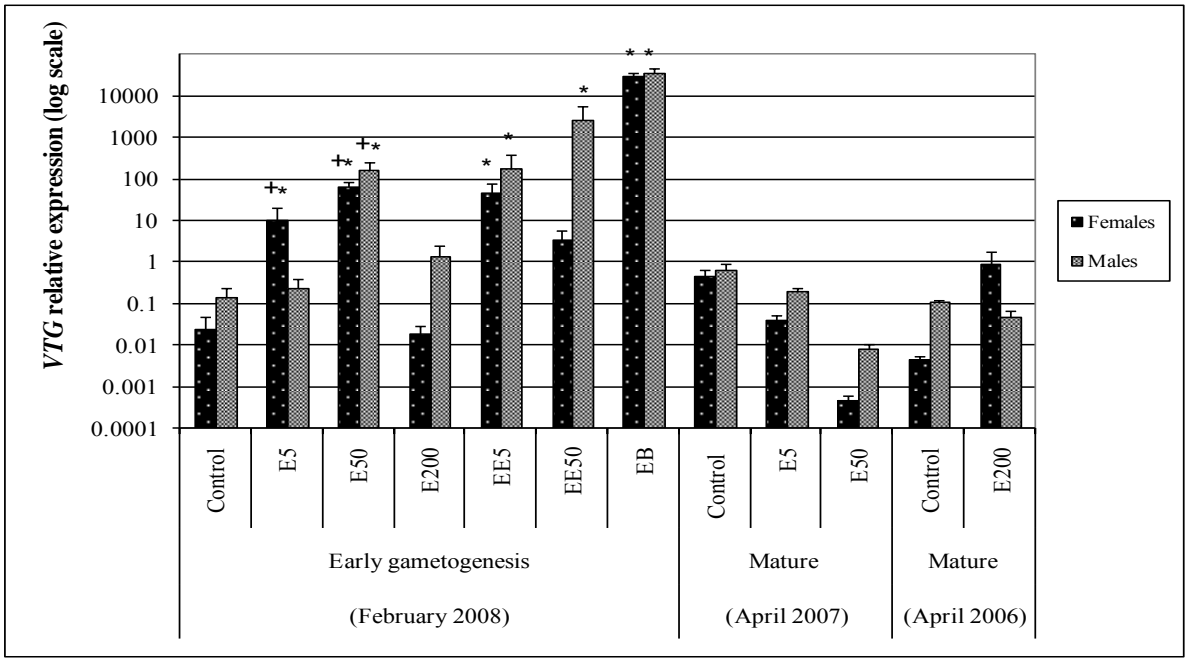


Figure 14: *VTG* relative mRNA expression in *M. edulis* gonads during early and late gametogenesis stages. C=control mussels; E5=mussels exposed to 5 ng/l E2; E50= mussels exposed to 50 ng/l E2; E200=mussels exposed to 200 ng/l E2; EE5=mussels exposed to 5 ng/l EE2; EE50=mussels exposed to 50 ng/l EE2; EB=mussels exposed to 200 ng/l EB. Mean data plotted ± SEM. n=10. Pair wise comparisons (between exposed/control females; exposed/control males) were carried out using the non-parametric Mann-Whitney U test and $P<0.05$ significance. Treatments marked with * are significantly different ($P<0.05$) from their corresponding control. Treatments marked with + are significantly different from their counterpart at mature stages. Treatments market with ^ are significantly different from their opposite sex, same exposure regime. Note log scale.

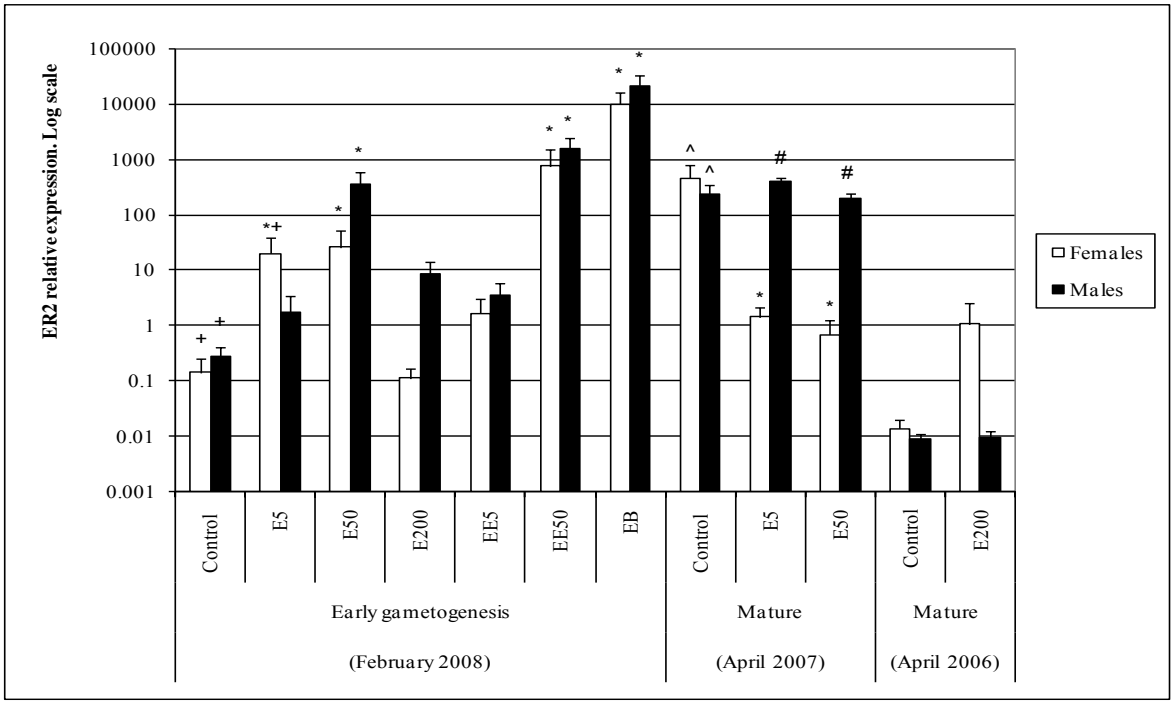


Figure 15: *ER2* relative mRNA expression in *M. edulis* gonads during early and late gametogenesis stages. C=control mussels; E5=mussels exposed to 5 ng/l E2; E50= mussels exposed to 50 ng/l E2; E200=mussels exposed to 200 ng/l E2; EE5=mussels exposed to 5 ng/l EE2; EE50=mussels exposed

to 50 ng/l EE2; EB=mussels exposed to 200 ng/l EB. Mean data plotted ± SEM. n=10. Pair wise comparisons (between exposed/control females; exposed/control males) were carried out using the non-parametric Mann-Whitney U test and $P<0.05$ significance. Treatments marked with * are significantly different ($P<0.05$) from their corresponding control. Treatments marked with + are significantly different from their counterpart at mature stages (April 2007). Treatments marked with ^ are significantly different from their previous year counterpart (April 2006). Treatments marked with # are significantly different from their opposite sex, same exposure regime. Note log scale.

The results confirm previous observations regarding the expression levels of serotonin, COX and CYP3A, indicating a possible involvement of steroids in the sexual maturation of mollusc species. Together, these data suggest that estrogen may have an impact on reproductive processes in bivalves. Based on these observations, the aim of our next study was to adopt an exploratory approach to identify novel genes differentially expressed in the process of maturation and response to estrogen in the marine bivalve, *M. edulis*.

New molecular techniques (SSH = suppressive subtraction hybridization) were used to highlight the cellular pathways affected by estrogen exposure, then, by targeting key genes involved in these pathways, measurements have been performed to assess the potential of these biological indicators to alert on changes in the mollusc endocrine system. The use of SSH in gonad samples at different stages of gametogenesis and (in parallel) after an exposure to estrogen in controlled conditions has allowed to identify several differentially regulated genes, including the gene associated with the androgen receptor, lysine and the yolk sac of an envelope sequence (Tables 4 and 5). These sequences provide a new path for studying and understanding the role of sex hormones in invertebrates and might lead to the identification of keys biomarkers of endocrine disruption.

Table 4: Differentially expressed genes in testis of *M. edulis* at different stages of the reproductive cycle and following experimental exposure (50ng/l E2)

Clone accession no.	Category & gene identity	Length (bp)	Homolog species /Accession no.	*E*-value
Down-regulated in early developing testes relative to mature testes				
Cellular component/Apoptosis/Cell cycle				
HQ690234	Senescence-associated protein	155	*Brugia malayi* XP_001900327.1	$5.0E^{-10}$
AJ492924.1	Histone 2A	301	*M. edulis* AJ492924.1	$1.0E^{-108}$
AY484747.1	16S ribosomal protein	931	*M. edulis* AY484747.1	0
Signalling				
FM995162.1	Vitelline coat lysin M7 precursor	635	*M. edulis* BAA03551.1	$3.0E^{-123}$
HQ678182	Sialic acid binding lectin	397	*Helix pomatia* ABF00124.1	$4.0E^{-15}$
Up-regulated in early developing testes relative to mature testes				
Cell cycle/Spermatogenesis				
HQ678180	Testis-specific serine/threonine kinase 1 (TSTK1)	815	*Strongylocentrotus purpuratus* XP_787865.1	$4.0E^{-70}$
HQ678181	Testis-specific A-kinase-anchoring-protein	182	*Gallus gallus* XP_002162537.1	$9.0E^{-6}$
HQ67816	Histone H2A isoform 2	332	*Haliotis discus discus* ACJ12611.1	$1.0E^{-53}$
HQ678184	Beta-tubulin	511	*C. gigas* AAU93877.1	$9.0E^{-81}$
Signalling				
AB257133	ER	111	*M. edulis* BAF34366.2	$1.0E^{-12}$
HQ678183	Bindin precursor 5 repeat variant (acrosomal protein)	392	C. gigas ABQ18234.1	$7.0E^{-14}$
HQ678185	Phosphodiesterase 1	533	S. purpuratus NP_001091918.1	$5.0E^{-62}$
Mitochondrial electron transport				
AY130198.1	Cytochrome c oxidase subunit III	254	M. edulis AAV68300.1	$2.0E^{-31}$

Six *target* mRNAs were selected for qPCR validation of the SSH differential expression results (Fig. 16 and 17). Both *vitelline coat lysin precursor* mRNA and *sialic acid binding lectin* were statistically significantly differentially regulated according to testis stage of maturity, up-regulated as the testis mature. Conversely, *testis-specific serine/threonine kinase 1 (TSTK1)* and *ER* mRNA expressions measured using qPCR are statistically significantly down-regulated in mature testis. *Cytochrome b* mRNA expression was statistically significantly down-regulated in E2-exposed mussels relative to control samples, again confirming the SSH result. Also, *RWD domain containing protein*

4A, highlighted by SSH as down-regulated in control mussel testis samples relative to E2-exposed samples, was identified using qPCR as down-regulated in early developing testis samples relative to mature samples.

Table 5: Differentially expressed genes in testis of *M. edulis* at different stages of the reproductive cycle and following experimental exposure (50ng/l E2).

Category/gene identity	Length	Homologue species
Protein binding/Apoptosis/Cell cycle		
Complement C1q like protein	111	*Ailuropoda melanoleuca*
Alpha tubulin	297	*C.gigas*
Beta tubulin	501	*Rattus norvegicus*
Ribosomal protein L7	240	*C.gigas*
Bromodomain adjacent to zinc finger protein	369	*G.gallus*
Elongation factor 1 gamma	162	*Saccoglossus kowalevskii*
Ferritin like protein	492	*Pinctada fucata*
Senescence associated protein	318	*Trichoplax adhaerens*
Spectrin beta chain	293	*Harpegnathos saltator*
Vitelline envelope zona pellucida domain 9	900	*Haliotis rufescens*
Signalling		
C1q domain containing protein	141	*Argopecten irradians*
Rwd domain containing protein 4A (small androgen receptor)	240	*Caligus regercresseyi*
Electron transport		
Hemagglutinin/amebocyte aggregation factor precursor	240	*Salmo salar*
Cytochrome c oxidase subunit II	448	*M.edulis*
NADH dehydrogenase subunit I	647	*M.edulis*
Triosephosphate isomerise TIM	156	*Metapaeneus ensis*
Cytochrome c oxidase subunit I	534	*M.edulis*
Cytochrome b	302	*M.edulis*

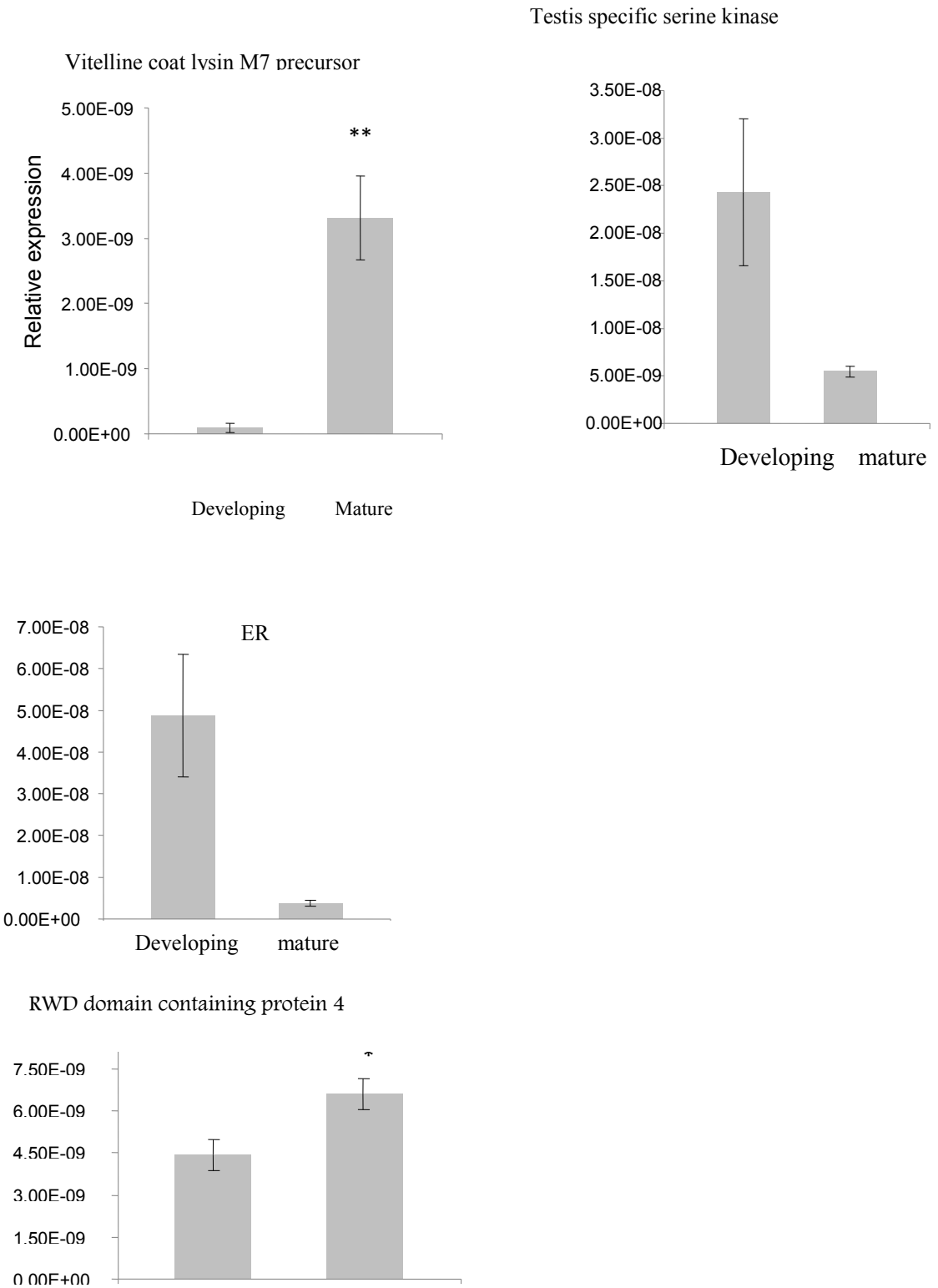


Figure 16. Targeting key genes involved in endocrine disruption processes to use as environmental monitoring analytical tools. Real-time quantitative RT-PCR validation of differential screening results of *M. edulis* developing gonad versus mature gonad samples. Data plotted as mean ± SEM, n=15 samples. *=p<0.05; **=p<0.01

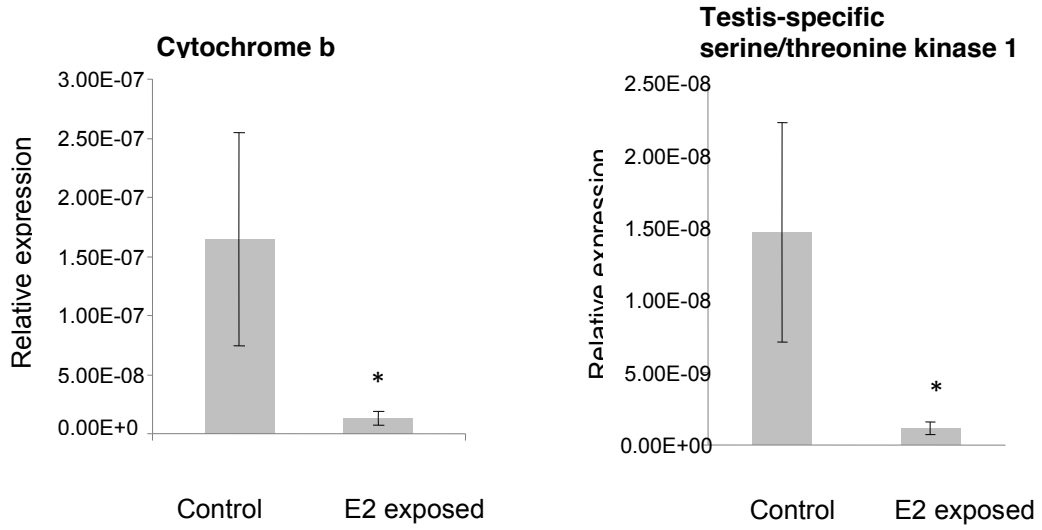


Figure 17: Real-time quantitative RT-PCR validation of differential screening results of *M. edulis* experimentally exposed to E2. Data plotted as mean ± SEM, n=15 samples. *=$p<0.05$; **=$p<0.01$.

Our results correlated with many lines of investigation indicate that steroids, particularly the sex steroids, play an important role in mussel reproductive control. For example, it appears that testosterone regulated processes may be controlled by additional mechanisms, involving receptor interacting proteins (i.e. Rwd), whilst some of the actions are likely mediated by receptors. In conclusion, our data concerning gene expression during spermatogenetic development and experimentally induced endocrine disruption give novel insights into previously uncharacterized aspects of molluscan reproductive pathways. We found forty seven potential genes whose transcript abundance fluctuates in coincidence with testis development/endocrine disruption processes. The data obtained allows a better understanding of mussel spermatogenesis and constitute a new focus for invertebrate endocrinologists in planning further experiments.

III.3. Highlighting cell pathway involved in the feminization of male clams (*Scrobicularia plana*)

In parts of the DIESE Interreg region, high incidences of intersex condition has been observed (see section III of this report), yet the causes are not known. Unlike vertebrates, sex determination and differentiation in marine bivalves depend less on sex chromosomes and more on hormones and environmental factors. Studies on complex effects of endocrine disruptors on bivalves are making a slow progress, partially because of poor understanding of mechanisms behind activation and impairment of sex and reproduction. *Scrobicularia plana* populations in UK have been shown to exhibit an extensive degree of intersex in the form of ovotestis. Mainly because of its estuarine habitat, *S. plana* is highly susceptible to estrogens, and still, the mechanisms behind these endocrine disruption effects remained unexplored.

The suppression subtractive hybridization (SSH) method was used in our laboratory to identify differentially expressed transcripts in the gonads of adult clams exhibiting various degree of intersex. Transcripts have been identified in forward and reverse subtracted libraries, laying the foundation for screening and cloning new and specific genes involved in endocrine disruption in bivalves.

Subsequently, quantitative real-time polymerase chain reaction (qPCR) was used to measure expression of genes that were differentially-expressed in the SSH analysis.

Several interesting mRNA transcripts were identified and validated as down-regulated in clam intersex samples. These encode a variety of proteins involved in cell signalling (RACK1), cell cycle (PCNA, histone H3), protein synthesis (ribosomal proteins), cytoskeleton (tektin), sperm physiology (tektin, SPL1) and energy metabolism (CYB, COX1). In several cases, the down-regulated response was more than 10 times less than the control males (Fig. 18).

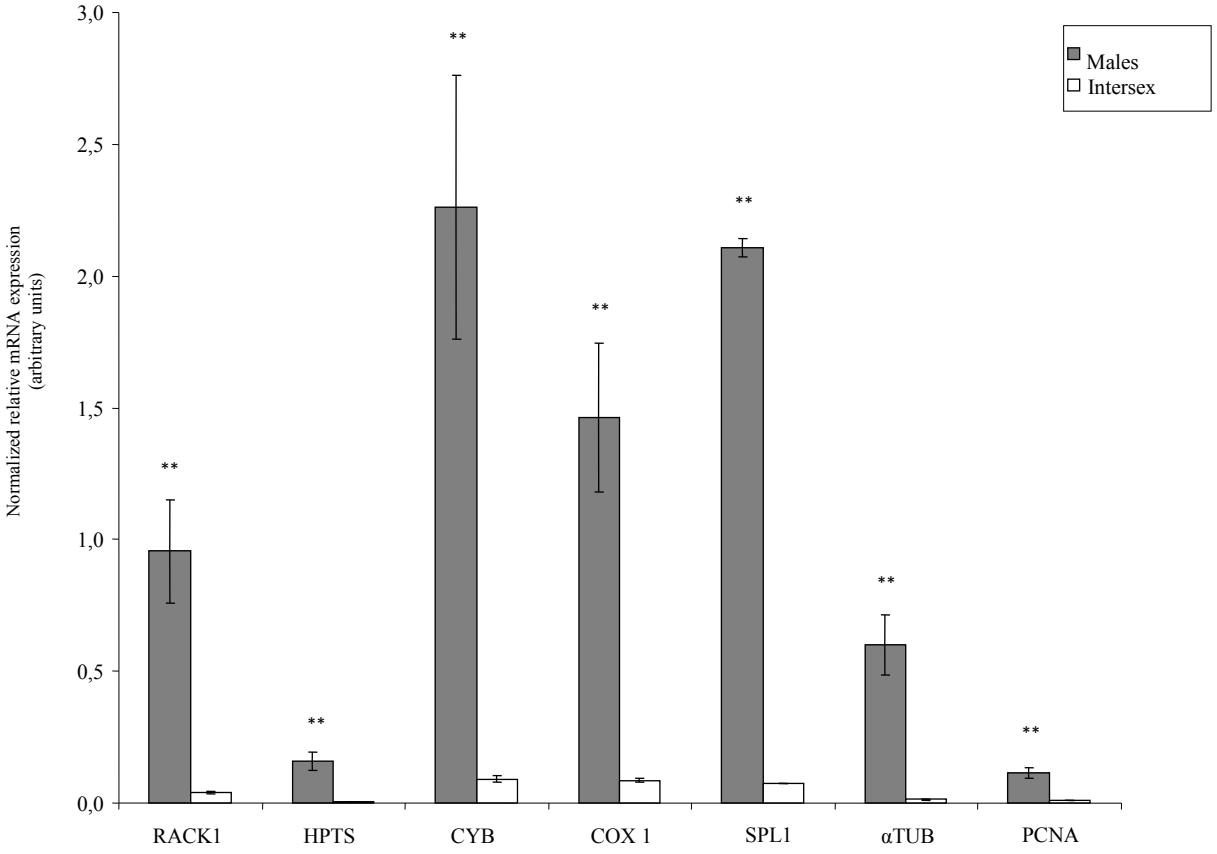


Figure 18: qRT-PCR validation of differential screening results of *S. plana*. Histogram bars represent normalized average relative mRNA expression ± standard error of the mean. ** indicates significant differences ($p < 0.01$; $n = 10$) of mRNA expression between male and intersex samples

RACK1 is a scaffold protein that interacts with kinases and plays a central role in signalling. A previous study reported that RACK1 interacts with androgen receptor (AR), enabling its translocation from the cytoplasm to the nucleus, and thus controlling transcription of AR target genes. RACK1, along with PCNA, were the most commonly sequenced transcripts down-regulated in intersex clams. PCNA is involved in cell cycle at the DNA synthesis stage, acting as a 'DNA clamp' in the replication process. Studies using Pacific oyster, *C. gigas*, have shown that in the gonadal tubules, *PCNA* expression is elevated in males compared with females, with a high and constant level

during the initial stages of spermatogenesis. Here we observe *PCNA* down-regulation associated with intersex condition, reflecting the female pattern for oyster. Histone H3, down-regulated in intersex clams, is also involved in cell cycle by controlling different phases of cell division in mitosis and meiosis. Combined, *RACK1*, *PCNA* and *histone H3* down-regulation suggest changes in cell signalling and cell cycle are associated with the intersex condition.

Two proteins involved in sperm physiology (tektin and SPL) were also identified as down-regulated in intersex males relative to normal male clams. Tektin is involved in sperm mobility in mice and studies have shown that tektin-deficient mice are subfertile. In molluscs, a search of EST sequences in NCBI databases revealed that an oyster tektin3 homolog was reported as down-regulated in oysters experimentally exposed to mercury (GenBank accession no. CD648637, unpublished). SPL1 has previously been isolated from jackknife clam, *Ensis minor*, yet its role in sperm physiology has yet to be elucidated.

The next step was to validate the results by analysing the gene expression in clams from natural populations and target key genes which can be used as environmental monitoring analytical tools. Following the discovery of intersex in *S.plana* in the western Channel, an intensive programme of fieldwork was undertaken on both sides of English Channel (La Manche). The aim was to sample *S. plana* populations and establish sex ratios, and the frequency and severity of intersex as indicators of feminisation/demasculinization. Intersexed individuals were identified in 77% of populations analysed in UK and 70% of French ones (although the total no of stations on French side was relatively small).

Sediments and clams were collected for chemical analysis to establish if there is a link between the occurrence of intersex in clams and contamination. Initial results indicate high levels of antiandrogenic activity at many sites, indicating widespread contamination of chemicals that have the potential to demasculinize aquatic biota.

III.4. Identifications of mechanisms of immunotoxicity in the sea bass, *Dicentrarchus labrax*

III.4.1. Introduction

With increasing awareness of the role of environmental stressors in childhood diseases and compromised adult immunity, researchers' attention has been driven recently to developmental immunotoxicity (DIT) (DeWitt *et al*., 2012; Luebke *et al*., 2006). Notably, an increase in asthma and allergic diseases in children coupled with the documentation of the impairment of the developing endocrine system, nervous system and immune system induced by environmental chemicals lead to proposals for DIT risk assessment (Bigsby *et al*., 1999; Bilitewski, 2008; Dietert and Zelikoff, 2008; Holsapple and O'Lone, 2012). In particular, the higher susceptibility to environmental pollutants during immune system ontology evoked interest in applying DIT, as such a phenomenon cannot be assessed by monitoring adult immune functions (Burns-Naas *et al*., 2008). Even though the ontology of the immune system is very complex, requiring a careful orchestration and regulation of the different stages, key developmental processes, and therefore "critical windows" can be identified species-independently during the vertebrate immune system development (Burns-Naas et al, 2008). However, these "critical windows" are predominantly based on defined events during the maturation of the adaptive immune system. Notwithstanding, due to the lack of basic knowledge on the developmental processes, this increased sensitivity of the developing immune system is not yet fully understood (Holsapple *et al*., 2012). It seems persuasive that with the establishment of safe exposure guidelines based on data from animals exposed during development, the adult immune system would be fairly protected as well. This is underscored by the results from exposures to diethylstilbestrol, diazepam, lead, 2,3,7,8-tetrachlorodibenzo-ρ-dioxin and tributyltin oxide, which represent a greater risk for the developing immune system, due to low dose action, a higher persistence or a combination of both (DeWitt *et al*., 2012; Luebke *et al*., 2006).

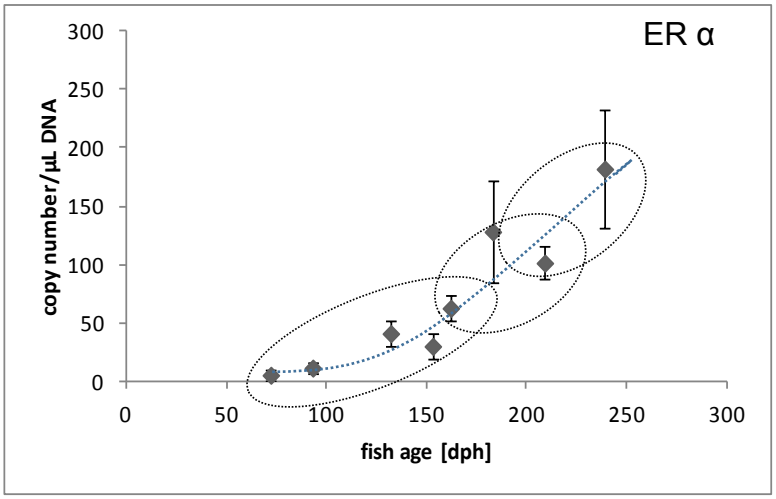
In line with this interest in this developmental immunotoxicity, studies on the sea bass immune system have been developed using juvenile fish in order to (1) provide new information on the development of the fish immune system and (2) unravel possible effects of estrogenic compounds during development. In addition, this work allow to study possible links between the immune and the endocrine system.

Two organs of the immune system received particular attention: the head kidney and the thymus. The head kidney, also referred to as anterior kidney or pronephros, is the mammalian bone marrow homologue and provides the compartment for hematopoiesis in teleosts. As major organ for B-cell development and proliferation, the head kidney is also responsible for phagocytosis, antigen processing, formation of IgM and the immune memory through melano-macrophage centres (MMCs) (Rauta *et al*., 2012). MMCs are the primitive analogues of the germinal centres of avian and mammalian lymph nodes (Agius, 2003). The thymus is a paired organ and provides the basic microenvironment for the T-lymphocyte maturation and proliferation. It is divided into a cortical and a medullary compartment and consists of cortical and medullary epithelial cells, macrophages, dendritic cells and mesenchymal fibroblasts and, of course, T-cells at different maturation stages (Pallavicini *et al*., 2010). While the lineage specification and the assembly of antigen receptor genes develop during the cortical phase of the T-cells, subsequent differentiations are accomplished after T-cell migration to the inner part of the thymus, *i.e*. the medulla (Boehm *et al*., 2012). In general, T-cell development in bony fish is strictly associated with thymus organogenesis, due to the interaction with distinct structures of the different thymic compartments during the maturation process (Vadstein *et al*., 2013).

III.4.2. Development of the immune system

Results allow to characterize the time course of expression of the estrogen receptors in the head kidney. A continuous increase in α receptor expression was measured during development and up to 250 days post hatch (jpe) while the expression of β2 receptor increases sharply after 75 jpe and reaches a maximum after 180 jpe (Fig. 19), suggesting that estrogens are involved in the development of the anterior kidney and that the dedicated functions of the two receptors are different during

development. Similarly, the expression levels of various cytokines was measured. Levels of interleukin-1 β follows the same pattern as that of the β2 receptor with a peak of expression in the same period (i.e. 180 dph). Expressions of interleukin-6 and tumour growth factor (TGF-β) increased steadily during the development of the immune system (Fig. 20). Interestingly, the percentages of leukocyte populations changed dramatically after 180 dph (Fig. 21). A significant decline in the B lymphocytes population was recorded, probably due to migration of the B lymphocytes to other compartments such as the posterior section of the kidney or to the spleen where they can complete their maturation (Ye et al., 2011). This migration period also coincided with a very significant increase in macrophage activity of anterior kidney leucocyte populations (Fig. 22). Thus the period of development of near 180 dph appears as a particular period which can be very important for the immune system.



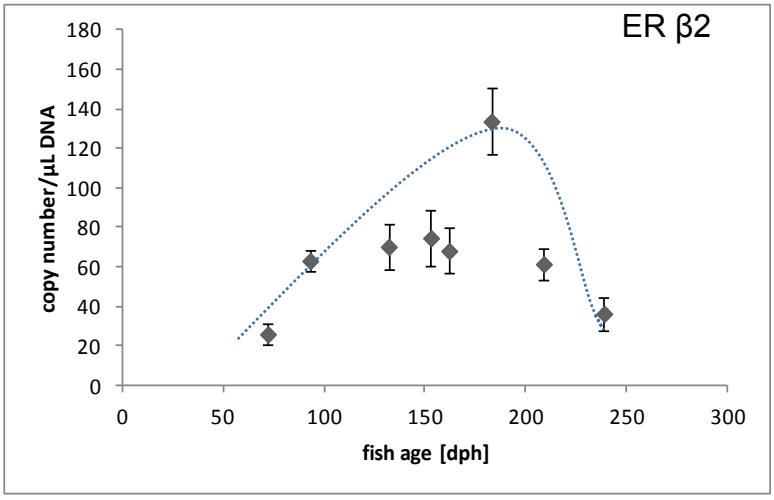


Figure 19 : ER α and ER β2 gene expression in the head kidney of sea bass at different days post hatching (dph) as copy number/µL DNA. means ± SE. n = 8-11.

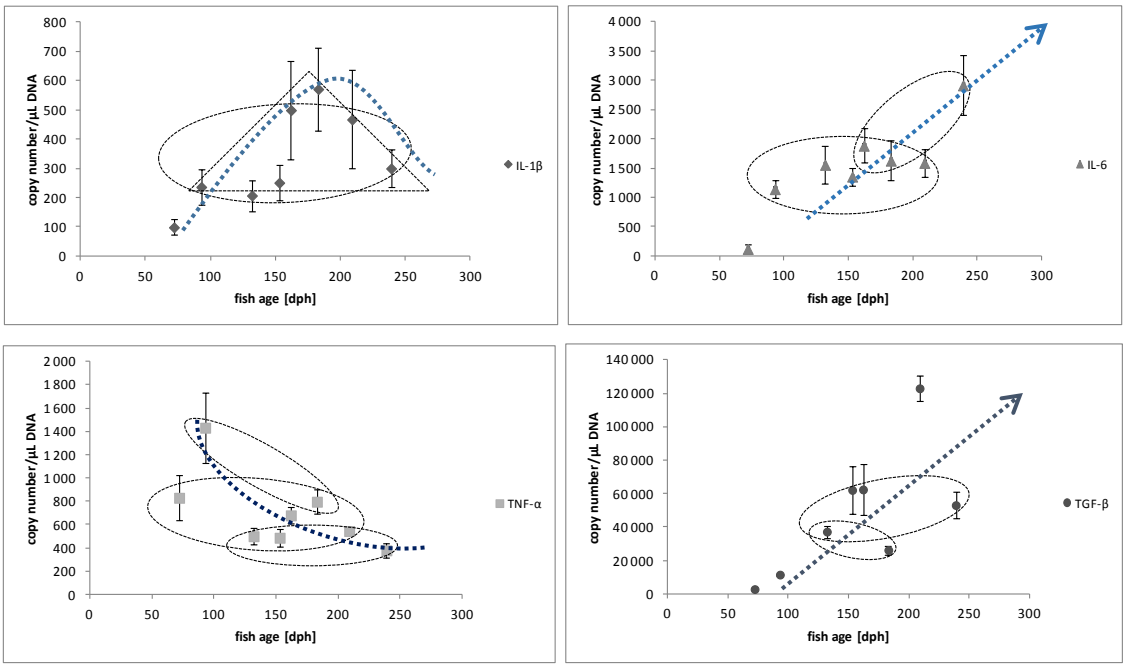


Figure 20: IL-1β, TNF-α, IL-6 and TGF-β gene expression (as copy number/µL DNA) in head kidney of sea bass at different days post hatching (dph). means ± SE; n = 7-11; single data points or data points in different circles indicate statistically significant differences.

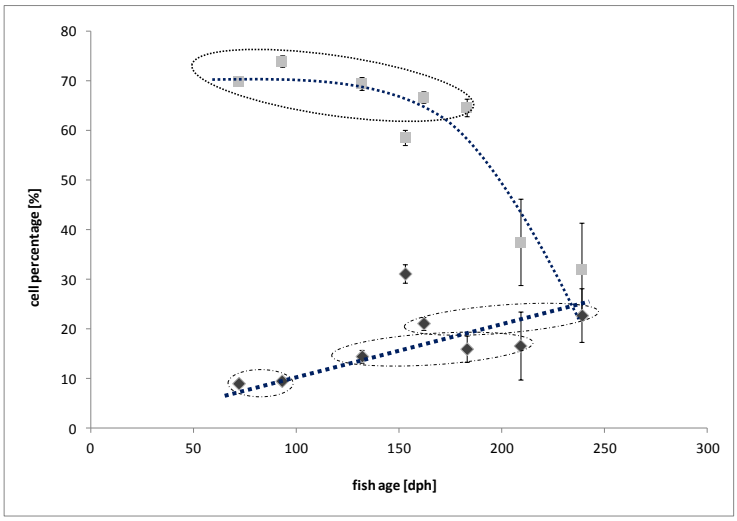


Figure 21: Development of head kidney leukocyte population with increasing fish age; the mean cell proportions (± SD) are indicated for monocytes/macrophages (diamonds) and lymphocytes (squares) of head kidney leukocytes; data points grouped in one circle indicate no statistically difference; n = 8-13

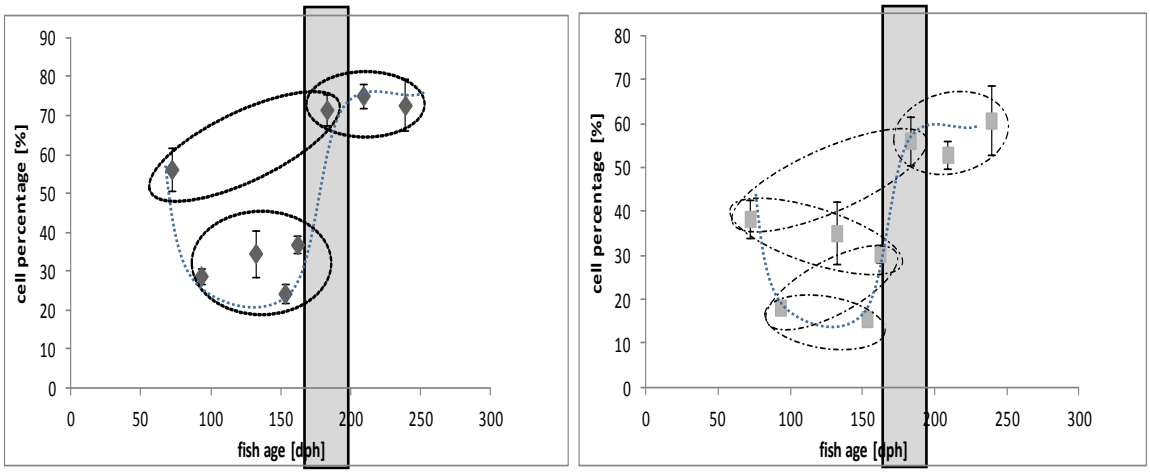


Figure 22: Phagocytic activity of monocytes/macrophages and lymphocytes at different ages; means ± SE; n = 8-13; data points grouped in one circle indicate statistically same groups.

III.4.3. Responses of the immune system to E2 exposure

Repeated exposures of juvenile sea bass to 20 ng E2/L were conducted under controlled conditions during the development of the immune system. Few significant changes were noted. However clear alterations in the expression of estrogen receptors were measured at the end of exposure periods for some groups of fish (Fig. 23). These periods ended at 162 and 183 dph *i.e.* during the critical period identified above. Similarly, exposure to estradiol resulted in a significant decrease in macrophage activity in the same periods (Fig. 24).

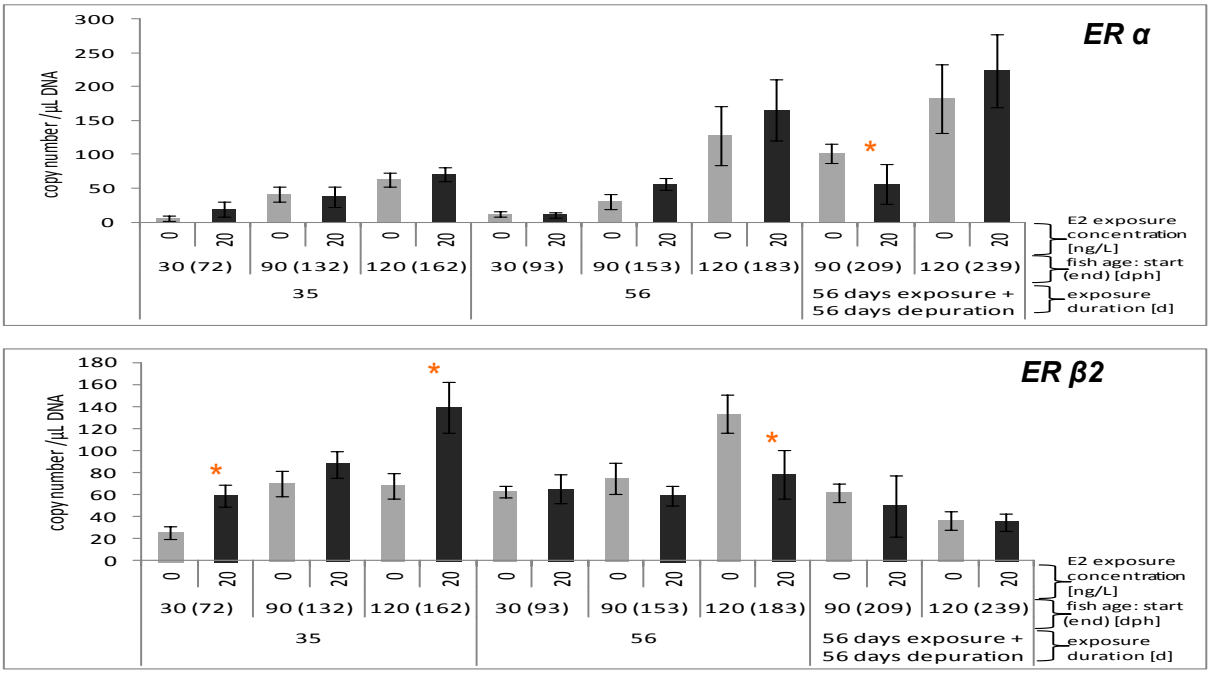


Figure 23: ER α and ER β2 gene expression as copy number/µL DNA in head kidney of control (light grey bars) and 20 ng/L E2-treated (dark grey bars) *D. labrax*; means ± SE; n= 5-11; asterisks mark significant differences to non-exposed fish at the same age.

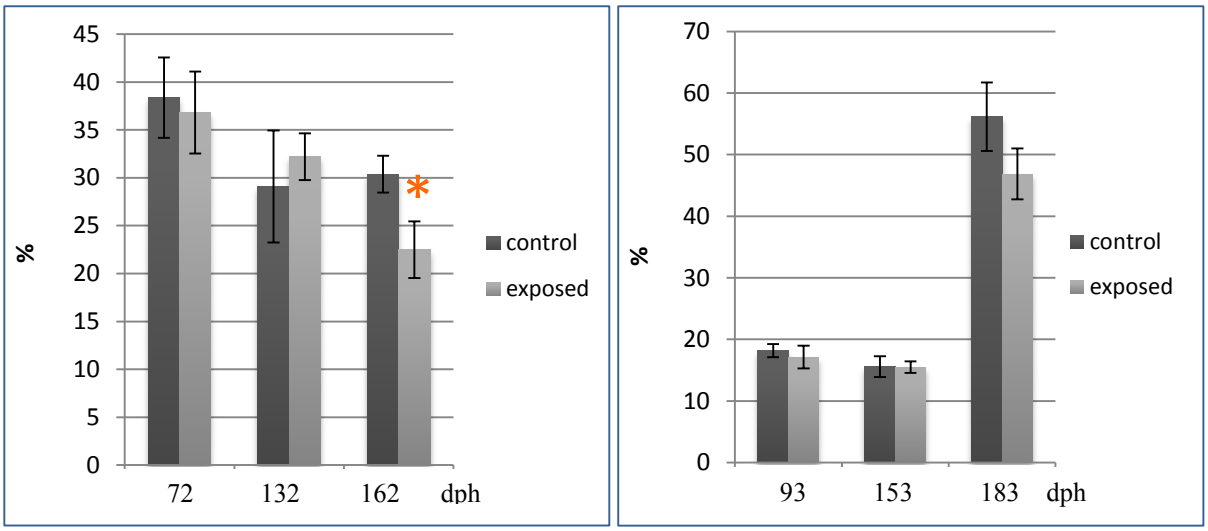


Figure 24: Phagocytic activity of leukocyte populations from the sea bass anterior kidney after 35 (A) and 56 (B) days of exposure to 20 ng E2/L during development. The activity was measured at the end of the exposition period, either at 72, 132, 162 days post-hatch (dph) after 35 days of exposure or at 93, 153 and 183 days post-hatching after 56 days of exposure . The bars indicate the mean ± standard error. n = 8-13. Points grouped in a circle are not significantly different.

A histological study was conducted to document the development of the thymus in the sea bass. Results showed, in agreement with the literature, that the thymus regionalized into cortex and medulla after 60 dph (Fig. 25) and developed further steadily during development. However exposure to 20 ng / L estradiol resulted in a highly significant enlargement of the organ and its two compartments in see bass between 90 and 153 dph, that is to say during an important period of maturation of T lymphocytes (Fig. 26).

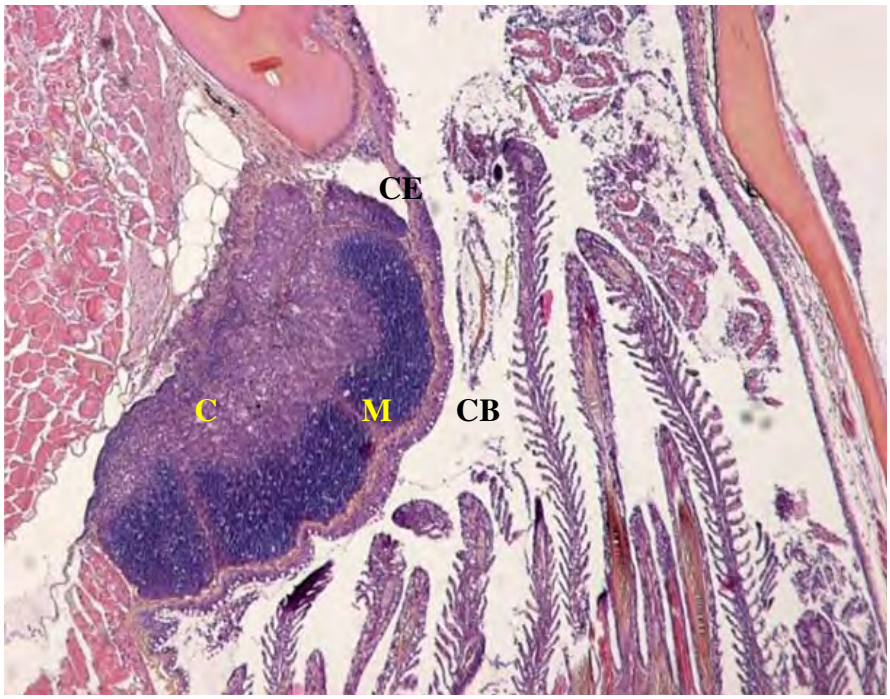


Figure 25: Transversal cut of the thymus of 60 dph *D. labrax*, differentiated in cortical (C) and medullary (M) region; GC = gill chamber; EL = epithelial cell layer; Haematoxylin-Eosin staining; magnification: 50x..

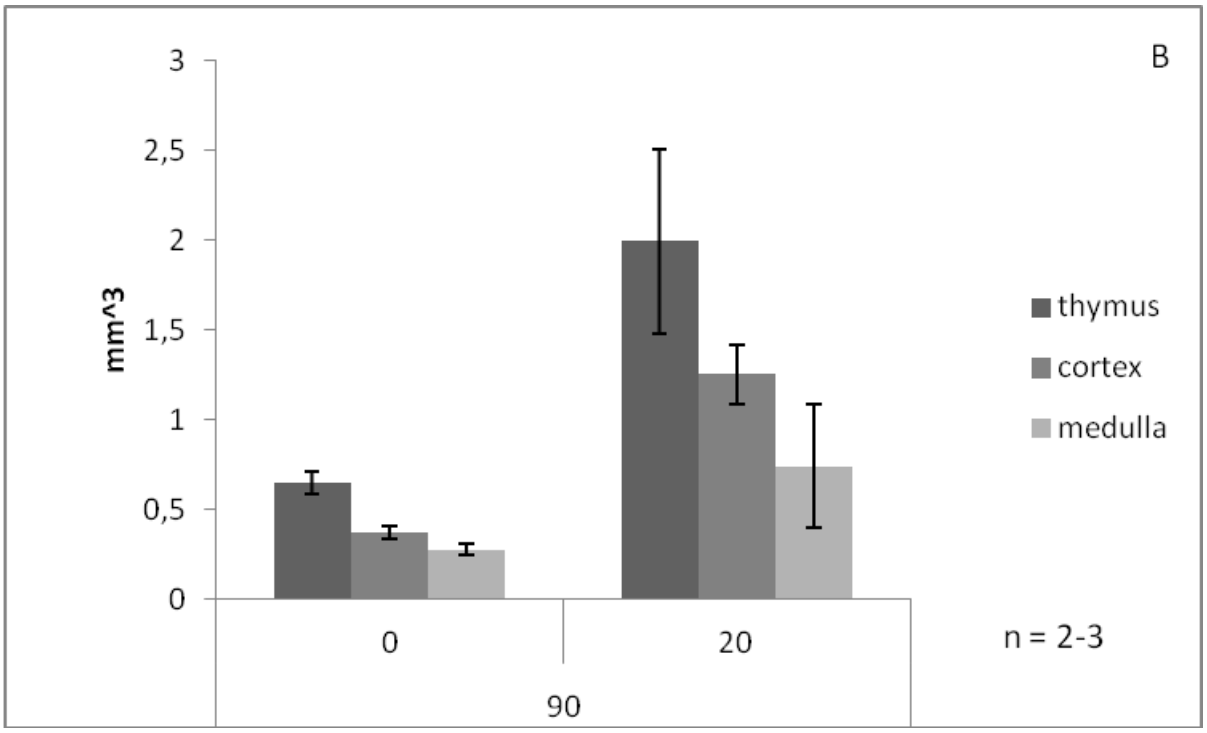


Figure 26: Thymus, cortex and medulla volume of 90 dph sea bass exposed for 56 days to 0 and 20 ng/L E2; means ± SE.

In conclusion, this work identified a critical period of development and of sensitivity to estrogen exposure of the sea bass immune system. This particularly sensitive window must be taken into account to better assess the risks associated with the presence of xenobiotics in the development of the immune system.

III.5. Identification of an androgen-regulated mechanism in the bullhead (*Cottus sp.)*

It has been reported a marked sexual dimorphism in renal tissue during the breeding season of the bullhead, *Cotius cotius* (Fig. 27). This dimorphism is due to a change in the kidney of male fish associated with the hypertrophy of renal tubules and the secretion of syaloglycoproteins as highlighted by immunohistochemistry (Bucher and Hofer, 1993, Hentschel, 1982). However, there is no information regarding the androgen inducibility and function of this renal secretion. A preliminary experiment conducted at INERIS showed increased nephro-somatic index, histological changes in the kidney and secretion of acid and neutral mucopolysaccharides in bullhead exposed for 7 days at 20 mg/L 11-ketotestosterone. These preliminary results require confirmation but suggest that renal hypertrophy and synthesis of syaloglycoproteins might be inducible by androgens. Thus, this species is likely to provide a novel biomarker of androgenicity. To identify this marker, the first step was to identify biochemical and/or histological androgen-regulated signals. Then, the identified signals were characterized by laboratory and field experiments in sampling sites known to be contaminated with molecules with androgenic activity (Sanchez et al., 2008).

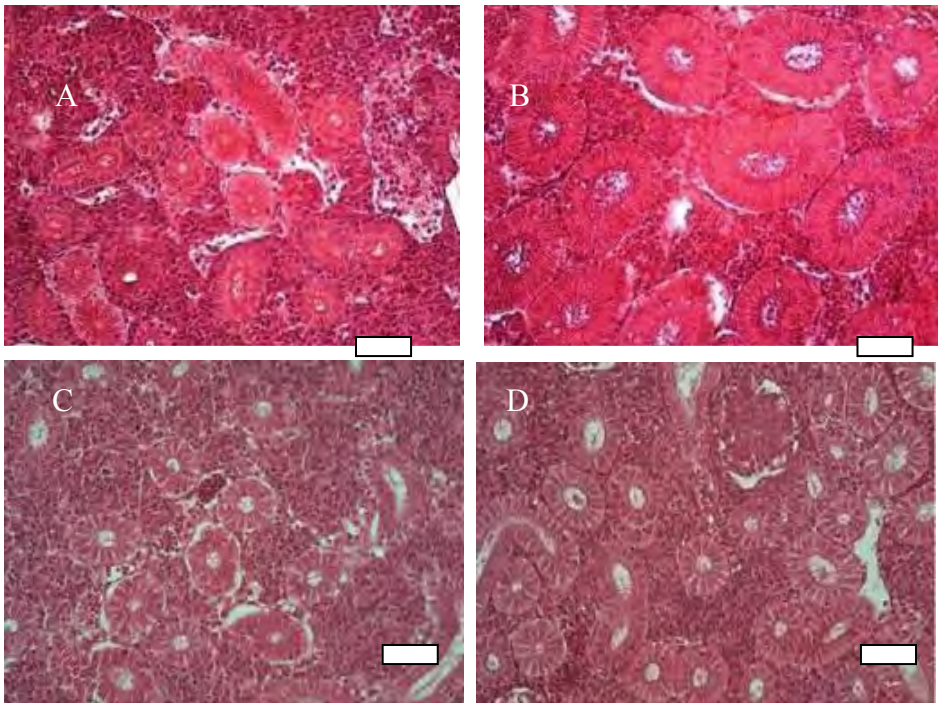


Figure 27: Transversals sections of female (A) and male (B) bullhead head kidney during the breeding period, and of female (C) and male (D) bullhead head kidney outside of the breeding period. Bars indicate 30 µm.

In order to check whether the renal tubule hypertrophy and the synthesis of syaloglycoproteins could be inducible by androgens, bullhead sampled from the field and acclimated for about two months in the laboratory were exposed to different concentrations of different reference androgenic molecules (DHT, trenbolone) dissolved in dimethyl sulfoxide (DMSO 0.001% per tank) or to the solvent only. During the exposure, 10 fish per tank were placed in aquariums containing 4L of water. After exposure, the fish were sacrificed, measured, weighed and sexed. The kidneys were removed and weighed in order to develop a histological marker, i.e., the KEH (Kidney Epitelium Height) available in the stickleback (Borg, 1993).

In all experiments KEH values obtained for males and females did not differ from each another. Thus the results are presented as a pool of values in both sexes.

No difference was observed between KEH of bullhead exposed to DHT and control fish. Indeed, the average height was 16 microns ± 1.05 for controls and 16.53 ± 0.92 microns for fish treated with 5 mg / L DHT (Fig. 28).

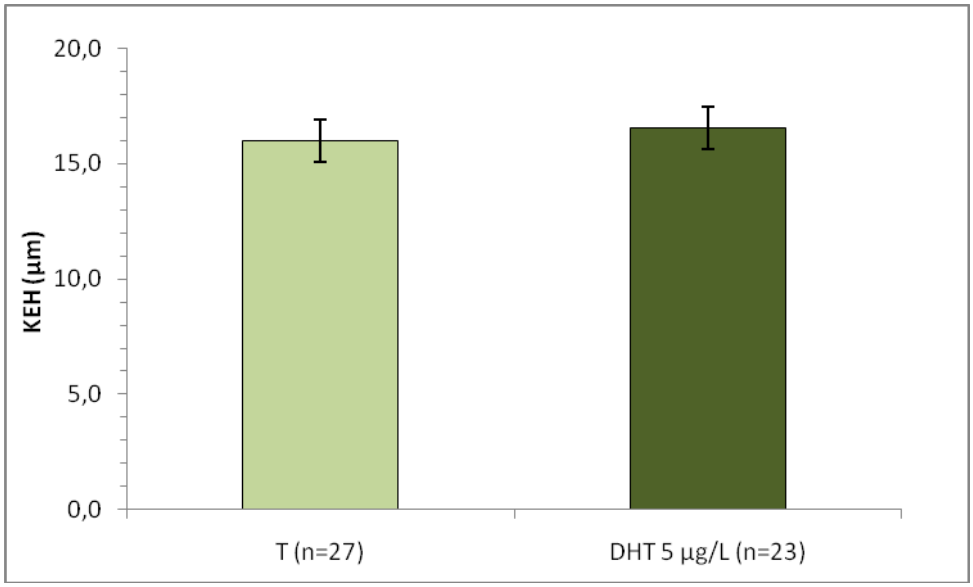


Figure 28: KEH (Kidney Hepithelium Height) bullhead of exposed to dyhydrotestostérone (DHT) for 21 days or to solvent DMSO (T). Data are presented as mean ± SD.

It is interesting to speculate on the mechanism of action of DHT in bullhead. Indeed, several publications have highlighted the power of the hormone androgen. It is indeed a biologically active metabolite of testosterone, formed under the action of the enzyme 5-α-reductase. It has been shown that exposure to DHT to measured concentrations greater than or equal to 6 mg/L for 14 days significantly increased the number of nuptial tubercles in both sexes of fathead minnow. (Panter et al., 2004). Similarly, in the stickleback was observed a dose-dependent response production of by spiggin exposing females to increasing DHT concentrations greater than or equal to 3 mg/L (Katsiadaki, et al., 2002a). However, some androgenic substances are more effective than others to stimulate renal hypertrophy in the stickleback. Thus, the 11-ketotestosterone appears to be more powerful than the 17α-methyltestosterone (De Ruiter and Mein, 1982) and dihydrotestosterone (Borg, 1993). The 17α-methyltestosterone itself is more powerful than dihydrotestosterone (Katsiadaki, et al., 2002a) and the 11-cétoandrostenedione more powerful than androstenedione (Andersson et al., 1988). However, the difference in power of androgens is not always related to the degree of affinity for the receptor and can other proteins interactions may be at play.

An exposure experiment was conducted using trenbolone. Indeed, the synthetic androgen is widely used in the United States as a growth promoter in cattle. This substance is stable in animal waste and is found in the aquatic environment through direct discharges, runoff or both. In addition, it has been shown that 17β-trenbolone was clearly androgenic *in vivo* to fathead minnow in waters containing 0.027 mg/L trenbolone in a 21-day exposure assay leading to the appearance of nuptial tubercles at the head of females (Ankley et al., 2003).

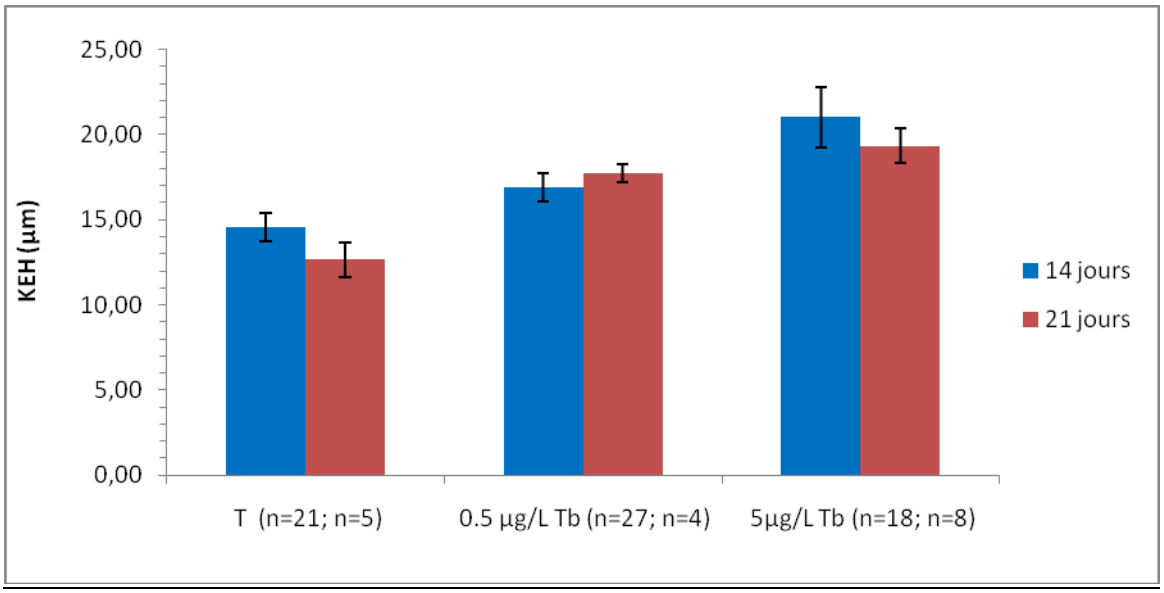


Figure 29 : Kidney hepithelium height (KEH) of kidneys from bullhead exposed to trenbolone (Tb) or solvent DMSO (T) for 14 days or 21 days. Data are presented as mean ± SD.

Bullhead exposed to trenbolone showed a dose-dependent renal hypertrophy in both exposure times (Fig. 28). In addition, for the same treatment there was no difference between the two exposure times. Thus, the synthesis of sialoglycoproteins appears to be effectively under androgen control.

To identify and characterize the kidney syaloglycoproteine induced by androgens, a proteomic approach based on two-dimensional electrophoresis and mass spectrometry was implemented to futures to develop and validate a specific quantitative ELISA. Kidney samples treated or not with trenbolone have been compared in order to identify variations in protein expression (Fig. 29).

After staining 9 spotted appeared differentially expressed in the exposed group (Figure 30). From these 9 proteins, three have been identified so far using mass spectrometry: the beta subunit of ATP synthase. (spot #1), the beta globin (spot # 9), and the nucleoside diphosphate kinase (spot # 8).

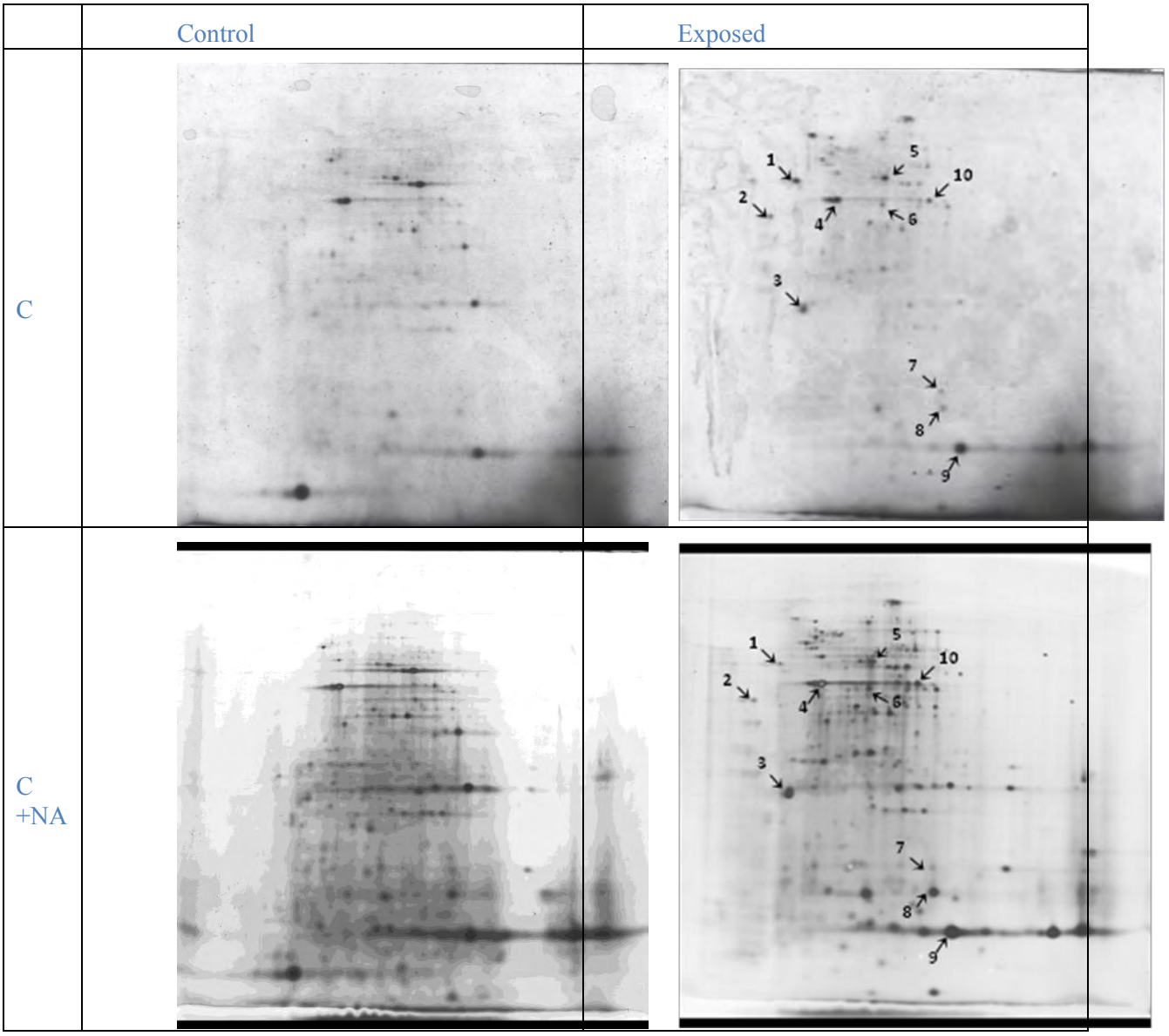


Figure 30: 2D gels analyses of protein extracts from female control (Control) or treated with 5µg/L trenbolone (Exposed) bullhead head kidney. Black dots were revealed using either coomassie blue (BC) or coomassie blue followed by silver nitrate staining (BC + NA).

III.6. Identification of endocrine disrupting effects in female roach (*Rutilus rutilus*)

It has been well established that a large number of man-made or natural chemicals occurring in the environment are able to disrupt the endocrine system of exposed organisms by interfering with their normal endocrine function. These compounds also called endocrine disrupting chemicals (EDCs) have been shown to cause alterations in development, reproduction, physiological homeostasis and health of vertebrates (Colborn et al, 1993). EDCs can modulate function, levels and distribution of endogenous hormones of exposed organisms by mimicking, antagonizing the actions of hormones, or by modulating hormone synthesis and metabolism (Segner, 2009).

Endocrine disruption has been reported to some degree in nearly all classes of vertebrates including mammals, birds, reptiles and fish (Tyler et al, 1998). As the aquatic system is considered as the final sink for most of the chemicals released in the environment, aquatic species including fish are common models used for the biomonitoring of ecosystems (Jobling and Tyler, 2003). Among the effects of EDCs, alterations of the reproductive system have been reported in different freshwater fish species all around the world with impacts such as abnormal plasma steroid and vitellogenin (VTG) levels, reduced spawning success, altered neuro-endocrine function, occurrence of oocytes in the testes of male fish, inhibited testicular and ovarian maturation (Kime, 1998; Vos et al, 2000; Van Der Kraak et al, 1992; Jobling and Tyler, 2003; Sumpter, 2002). The roach (*Rutilus rutilus*) a ubiquitous cyprinid fish in European freshwater ecosystems has been selected as a sentinel species for endocrine disruption biomonitoring in several studies (Tyler et al, 2007). Different alterations of the reproductive system have been shown for roach living in UK rivers (Jobling and Tyler, 2003), Danish streams (Bjerregaard et al, 2006), a Sweedish lake (Noaksson et al, 2001) and in French rivers (Minier et al, 2000; Maltret-Geraudie et al, 2008).

The Seine River in France constitutes one of the most polluted rivers in Europe with a basin characterized by a high population density (250 p.km^{-2} in average) and being highly impacted by 40 % of the French agricultural and industrial activities (Lafite and Romanã, 2001). As a consequence, the water and sediments of the Seine River shows high levels of contaminants i.e. nitrates, phosphates, heavy metals, polycyclic aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs) and pesticides such as triazine (Minier et al, 2006; Billen et al., 2001; Thévenot et al., 1998; Horowitz et al., 1999; Grosbois et al., 2006).Evidences of the presence of estrogenic compounds such as ethinylestradiol, estrone and nonylphenol have been found in fish and sediments of the Seine River (Peck et al, 2007; Minier and Amara, 2008). Roach sampled in the Seine River have been shown to be contaminated by organochlorine compounds including PCBs and synthetic pesticides (Blanchard et al, 1997; Guérit et al, 2008). Previous studies have also reported that male roach from the Seine River showed evidences of endocrine disruption (ED) with high intersex incidence, and altered levels of steroids and vitellogenin as well as impaired gametogenesis (Maltret-Geraudie et al, 2008; Geraudie et al, 2011). Most of the studies on ED in roach have been focusing on estrogenic responses in males, however, alteration of the female reproductive system might be more detrimental for the population. Accordingly, this study investigates the alterations of the reproductive system of female roach living in polluted areas of the Seine River.

Mature female roach *Rutilus rutilus* (>16 cm long) were collected in autumn 2010 from three different locations of the Seine river (Normandy, France) using nets or angling. The reference site, Venables (Ven) is an old sand quarry with low levels of contamination where no sign of endocrine disruption in roach has been found (absence of intersex fish, mean male plasma VTG concentration lower than 20 ng mL^{-1}; Geraudie et al., 2010a). The two other sampling locations, Poses and Elbeuf, correspond to anthropogenic areas with adjusted population equivalents of 442 and 11 respectively. Chemical analyses of these two impacted sites were carried out by the control survey network directed by the Seine water agency (Agence de l'eau Seine Normandie) and showed to contain a mixture of different polycyclic aromatic hydrocarbons, polychlorobiphenyls, polybrominated diphenyl ethers and phthalates.

The average length and weight of the fish from the 3 sampled sites was not significantly different as indicated in table 6. The Fulton's body condition factor (K) was similar for fish from all sites. However, the GSI was shown to be 1.6 and 1.7 times significantly lower for fish from the

contaminated sites (Poses and Elbeuf; 4.75 ± 0.58 and 4.48 ± 0.38) compared to fish from the reference site (Venables; 7.68 ± 0.63).

	Venables	Poses	Elbeuf
lenght (cm)	20.49 ± 0.5	18.63 ± 0.79	20.56 ± 0.84
weight (g)	104.09 ± 13.7	81 ± 16.94	110.58 ± 15.99
gsi	7.68 ± 0.63^{a}	4.75 ± 0.58^{b}	4.48 ± 0.38^{b}
fulkon's k factor	1.14 ± 0.02	1.08 ± 0.04	1.17 ± 0.04

Table 6: Biological parameters of female roach collected from the reference site (Ven) and the impacted sites (Elb and Pos) of the Seine River. Distinct letters indicate significant differences between groups ($p < 0.05$).

The maturation stages of the fish ovaries determined by histological observations are presented in figure 31. Most of the females fish from all sites showed vitellogenic gonads,i.e. with a majority of secondary oocytes. The percentage of fish exhibiting primary oocyte stages was higher in fish from the impacted sites (27 and 14 %) compared to fish from the reference site (5 %) thus showing a delay in maturation in these fish.

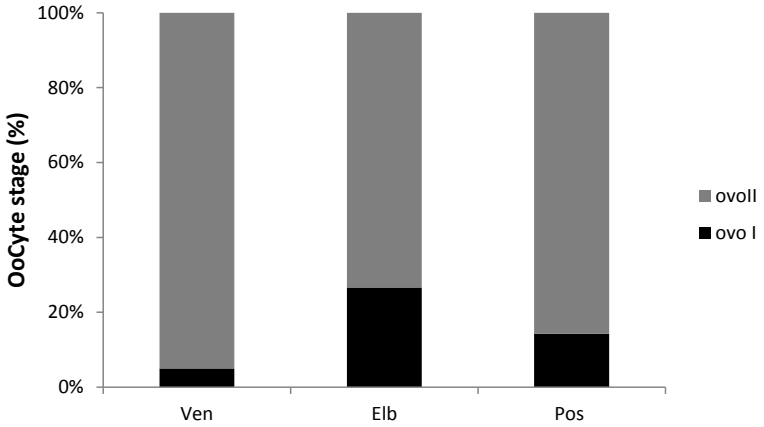


Figure 31: Ooctye stages of female roach collected from the reference site (Ven) and the impacted sites (Elb and Pos) of the Seine River. Ovo II is for secondary oocyte and Ovo I is for primary oocyte.

The diameter of secondary oocytes in fish from the different sampling locations was also recorded (Fig. 32). The fish from the two impacted sites, Elbeuf and Poses presented significantly reduced secondary oocyte diameters compared to the reference site fish: 589, 599 and 824 µm in mean respectively.

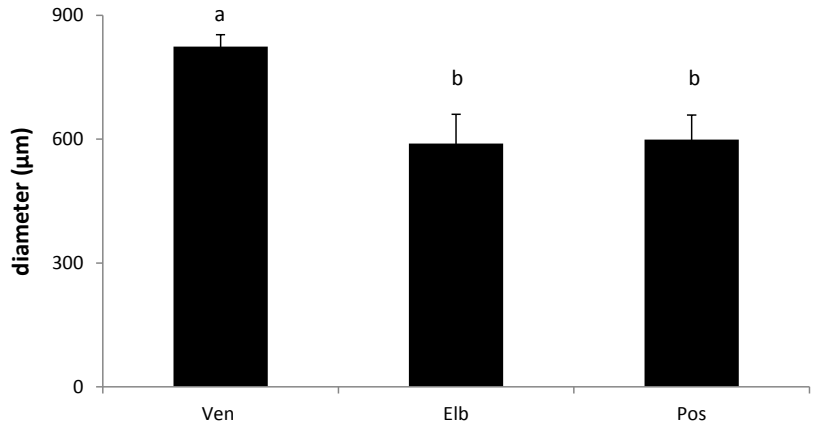


Figure 32: Oocyte diameter of female roach collected from the reference site (Ven) and the impacted sites (Elb and Pos) of the Seine River. Distinct letters indicate significant differences between groups ($p < 0.05$).

The plastmatic VTG levels were shown to be significantly lower for fish from the impacted sites compared to fish from Venables, the reference site (Fig. 33). The plasma VTG concentrations were 4 and 2 times lower in fish from Elbeuf and Poses respectively compared to fish sampled in the reference site.

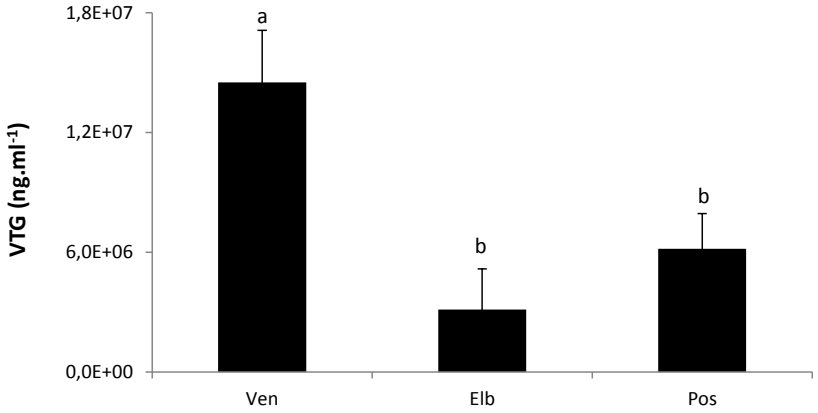


Figure 33: Plasma VTG levels of female roach collected from the reference site (Ven) and the impacted sites (Elb and Pos) of the Seine River. Distinct letters indicate significant differences between groups ($p < 0.05$).

For the aromatase activity, only fish with secondary oocytes were analysed (Fig. 34). The aromatase activity was significantly 3 times reduced in fish inhabiting Elbeuf and Poses (6.11 and 8.56 $pmol.h^{-1}.mg\ prot^{-1}$) compared to the fish from Venables with a mean of 16.75 $pmol.h^{-1}.mg\ prot^{-1}$.

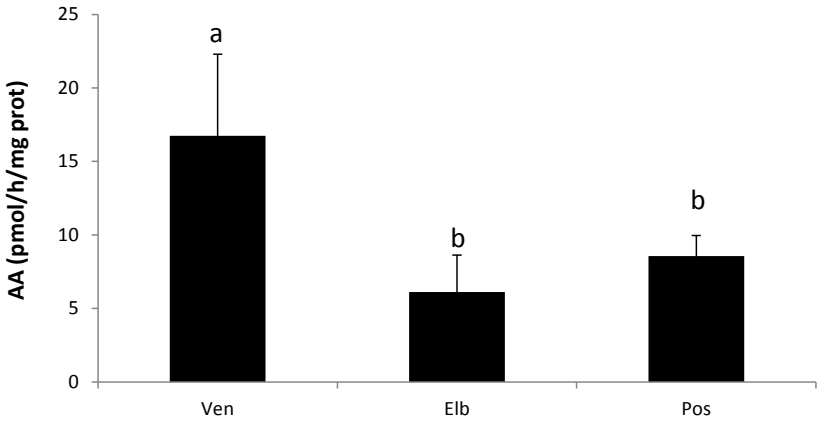


Figure 34: Gonadal aromatase activity of female roach collected from the reference site (Ven) and the impacted sites (Elb and Pos) of the Seine River. Distinct letters indicate significant differences between groups ($p < 0.05$).

In conclusion, this study reports several impairments of the reproductive system of female roach from the Seine River. The three sampling sites are located within a radius of 26 km and are subjected to the same weather conditions excluding the possible impact of abiotic factors on the gonad maturation of the fish. The size and weight of all fish from the different sites were similar indicating that a homogenous sample of maturing females was selected. This study gives additional information on the reproductive state of roach from the Seine River as other studies have already shown the impact of environmental EDCs on the reproduction of male roach (Maltret-Geraudie et al, 2008; Geraudie et al, 2011; Minier et al, 2000). Some of the observed alterations could lead to low fecundity and also alter the offspring survival and population health.

III.7. Integrated study using the flounder (*Platichthys flesus*)

III.7.1. Introduction

Over the past decade, there has been more integration of multiple biomarkers, allowing a better assessment of the quality of marine and estuarine waters in Europe (Hagger et al 2008; Thain et al 2008; Lyons et al. 2010). In this project, we focused our work on an estuarine fish, the flounder (*Platichthys flesus*) considered as a relevant sentinel species for monitoring water quality (Vethaak and Wester 1996; Stentiford et al. 2003, Lyons et al 2004. SGIMC 2011; Vethaak et al 2011; Laroche et al 2013). In this work, we considered a wide variety of biomarkers, taking into account the different levels of organization of living organisms, furthering our understanding of the responses of fish to chemical stress.

This study implemented a laboratory experiment to highlight the effects of complex cocktails of PCBs (polychlorinated biphenyls) and PAHs (Polycyclic Aromatic Hydrocarbons) on fish, alongside measures of biomarkers were performed on natural populations of flounder exposed to contrasting contaminations in selected estuaries.

The flounder, *P. flesus* (Fig. 35), is a euryhaline flatfish living preferentially on sandy mud and muddy estuarine and coastal areas (Déniel 1981). This species is widely distributed in Europe, from Norway to Portugal. The flounder is a catadromous fish, living most of the year in estuarine

areas and rivers, it will reach the ocean as adults to spawn. Along the French coast, spawning occurs during the winter (January to March). The laid eggs are pelagic, after 7 days, the larvae hatch and exhibit bilateral symmetry. The larvae metamorphose into juveniles approaching the coast, they will be recruited in the estuary in May. Flounder will remain 2 to 3 years in estuaries to reach a size between 110 and 250mm in the second fall, and with a large inter-individual variability in growth rates (Masson, 1987). During the winter, flounder will become sexually mature adults and spawn at the mouths of rivers or at sea, and give birth to the next generation.

Figure 35 : The flounder, *Platichthys flesus*

III.4.2. Biomarkers

Several biomarkers were used :

- DNA damages measured using the comet assay (in plasma)

The comet assay allows to measure single and double breaks (Lee et Steinert, 2003).

- Selected gene expression (in liver)

In response to chemical contamination, an living organisms may modify the expression of its genes to cope with the new situation. This change is often seen as an early response to chemical stress. Among the various genes whose expression is altered, cytochrome P450 1A (CYP1A) is particularly studied. It is involved in the transformation of hydrophobic xenobiotics into water-soluble metabolites and in the process of biotransformation of some toxic organic pollutants such as PAHs (Sarasquete and Segner, 2000). In simplified terms, the presence of contaminants in the body triggers detoxification process and therefore an increase in the expression of CYP1A gene. This process has been demonstrated in many fish species including flounder (Evrard et al. 2010a) and tomcod (Wirgin and Waldman 1998). Studies also showed regulation of the BHMT gene in the presence of contaminants in teleost (eg Lu et al, 2012; Yum et al, 2005.). BHMT could be involved in the detoxification phase II (Marchand et al., 2006). Natural populations of flounder living in contrasting environments showed differential expression of BHMT gene (Evrard et al, 2012; Marchand et al, 2006.).

- Biomarkers of the immune system

A major objective of this study was to explore the relevance of investigations on the immune system. The immune system is a set of cellular and humoral actors whose function is to maintain homeostasis, to protect the body from parasites, infections and malignant growths. It is therefore not a direct response to the presence of pollutants. Nevertheless, the presence of xenobiotics can weaken the immune system of a living organism, reducing its defense capabilities, for example to parasites (Dunier 1996 Dunier and Siwicki 1993). The fish immune system is very similar to that of higher vertebrates (Zelikoff, 1998). Fish have specific mechanisms of defense and non-specific, including both humoral and cellular responses.

The complement system has an important role in the innate immune system and involves about 35 membrane or soluble proteins (Holland and paneling, 2002). The functions of the complement are multiple: it is involved in the lysis of pathogens, the inflammatory response, it can stimulate phagocytosis or participate in the modulation of antibody production (DeFranco, Locksley and Robertson, 2009).

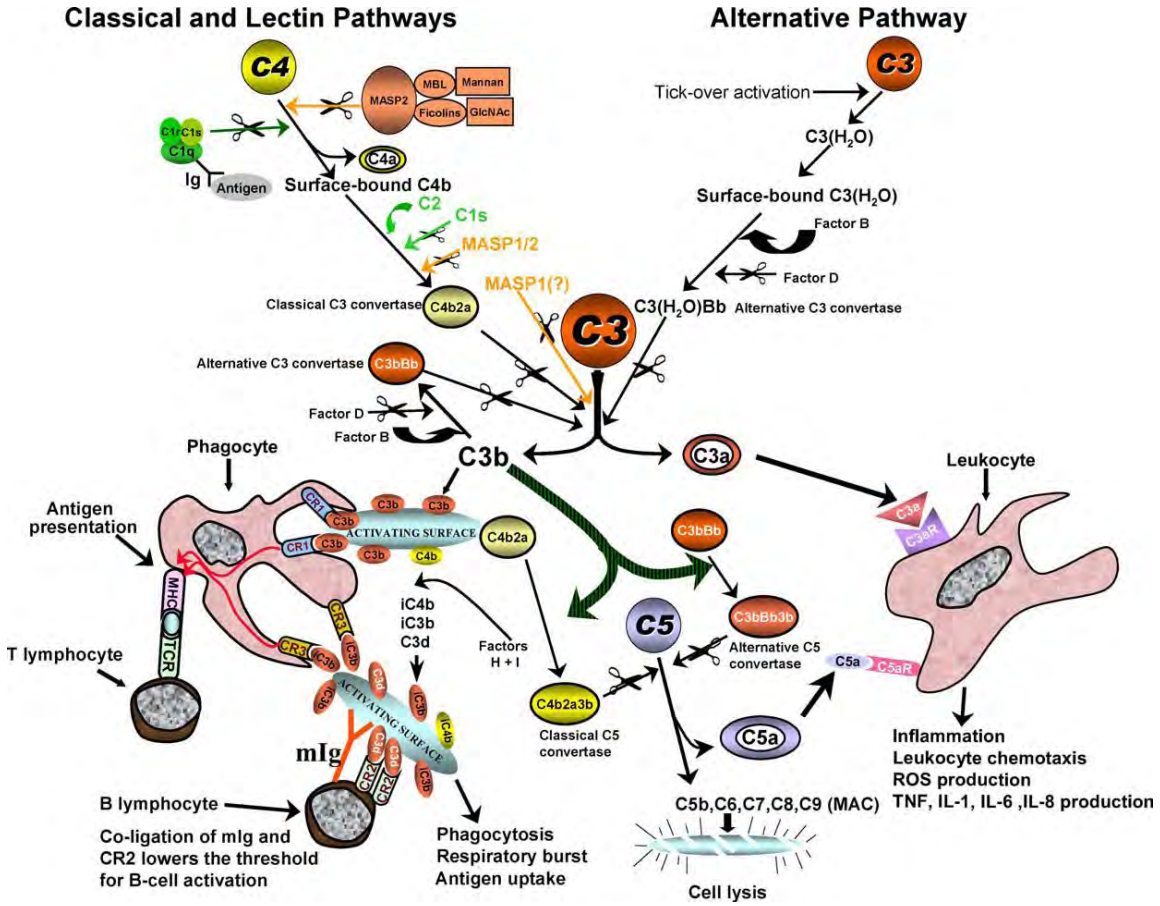


Figure 36 : Schematic view of the activation cascade of the complement (Boshra, Li et Sunyer, 2006)

TNF-α belongs to the family of cytokines. These are proteins produced by many immune cells and whose role is to control: (1) the intensity and duration of the immune response and (2) some process of differentiation and tissue proliferation (DeFranco, Locksley and Robertson, 2009). TNF owes its name to its cytotoxic effects on tumor cells. As the sequence of TNF-α has not yet been identified in our model species, we chose to study the expression of TNF-R which is the membrane TNF receptor (Smith, Farrah and Goodwin, 1994). TNF-R is involved in the regulation of apoptosis and inflammation (Glenney and Wiens, 2011). Measuring its expression allows to assess indirectly the modulation system of TNF. If a modulation of the expression of TNF-α is not necessarily associated with a modulation of the expression of its receptor, can still assume that a modulation of TNF-R system suggests a modulation of TNF in whole.

- Lysozyme activity (in plasma)

The lysozyme is an enzyme found especially in the lysosomes of neutrophils and macrophages, and is released into the blood. It is involved in the destruction of Gram-positive bacteria, with a lesser effect on Gram-negative bacteria by attacking the peptidoglycan present in the membrane of these cells (Skouras et al. 2003). It will thus cause cell lysis. Studies have shown that lysozyme activity was sensitive to the presence of contaminants in the environment (Bols et al., 2001a).

- Phagocytosis (head kidney)

Phagocytosis is one of the key mechanisms of the immune system. It occurs in several steps (Fig. 37). Initially the microorganism is recognized by chemotaxis by phagocytes, an immune cell that will completely cover the foreign body until it is internalized and degraded resulting in the formation of reactive oxygen species.

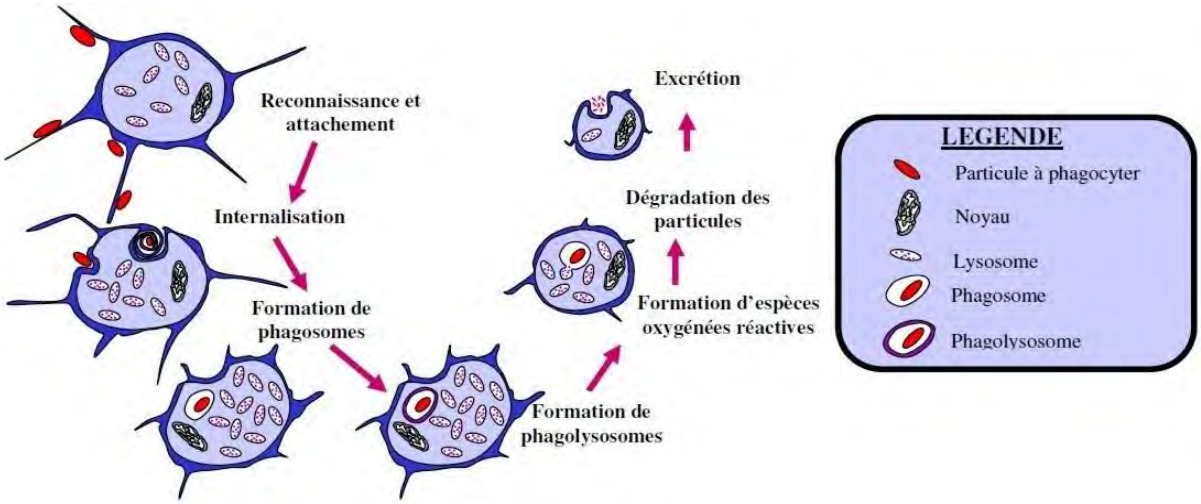


Figure 37 : les différentes étapes de la phagocytose (adapté de Bado-Nilles, 2008)

It has been shown, repeatedly, that in the presence of pollutants phagocytic macrophage activity could be reduced. This has for example been shown for plaice, *Hippoglossoides platessoides*, brought into contact with contaminated sediments, including PCBs (Lacroix et al., 2001).

- Energy Metabolism (muscle)

The metabolic rate of an organinism reflects the amount of energy it produces to ensure its vital functions. This activity can be considered as a biomarker indicative of the physiological status of fish (Plant et al, 2005). In the presence of xenobiotics, many physiological mechanisms are put in place: to maintain homeostasis, for detoxification, protection and/or repair of cells, all of these processes require energy. The evaluation of the overall energy production allows to estimate the cost of maintaining vital functions and the stress response (Beyers et al., 1999).

Some indicators of energy metabolism could be used as biomarkers in ecotoxicology. Cytochrome C oxidase (CCO) is the terminal enzyme of the mitochondrial respiratory chain and is responsible for 90% of the oxygen consumption of cells in mammals (Xu, 2005 Charles and Moncada). Measuring the activity of the CCO could be a good indicator of aerobic metabolism (Lapointe and Couture, 2010). Studies have shown that the presence of pollutants such as nickel or dispersed oil could be associated with increased activity of the CCO, so increasing the energy consumption (Cohen, Gagnon and Nugegoda, 2005, Lapointe and Couture, 2010).

- 2D proteomics approach (liver)

The study of the proteome is complementary to that of the transcriptome as the protein profile is influenced by the protein turnover (ie synthesis and degradation), post-translational modifications and their cellular compartmentalization. In this sense, proteins are more representative of the physiological effects and closer to the phenotypic response level.

There are many methods to study the whole proteome of an organism, or its subsellular fractions (Dial et al, 2012; Dowling and Sheehan, 2006; Martyniuk et al, 2011; Rabilloud et al., 2010; Monteoliva and Albar, 2004). All are based on common principles: the first step involves the extraction of proteins. These proteins are digested into peptide fragments (usually by trypsin) before ("shotgun proteomic", "top down approach") or after separation ("bottom up approach"). We distinguish gel based proteomic, using two-dimensional electrophoresis for protein separation and gel free proteomic for which the separation is done by chromatography. In some cases, proteins can be labeled before (fluorescence or isotopic labeling) or after separation (fluorescence, Coomassie blue, silver nitrate after 2-dimensional electrophoresis). This allows to identify proteins or peptides differentially expressed in a given condition.

Today, protein identification is mainly done by mass spectrometry (Cravatt et al., 2007). Various techniques exist. They are all based on a common principle:

-peptides are fragmented into volatile ions of different masses from a solution by ESI (electrospray ionization), or from a solid surface by MALDI (Matrix Assisted Laser Desorption Ionization).

-Ions are separated according to their mass and their charge (m/z) in the analyzer (by mass selection in a magnetic field created between four quadrupole electrodes, or by time of flight analyzer, (TOF);

-the relative abundance of ions according to their m/z is then detected, for generating the mass spectrum.

By using Tandem mass spectrometry (MS-MS), ions detected (precursor ions) are isolated and fragmented. The MALDI TOF-TOF allows fragmentation. It is then possible to determine the amino acid sequence of the protein, by comparing the spectra obtained with the theoretical fragmentation of peptides contained in the databases. We conducted these comparisons in silico using the MASCOT software and/or PEAKS, powered by databases of ESTs sequenced to date in flatfish. ESTs encoding proteins identified were then re annotated by homology using BLAST (Basic local alignment search tool) on the basis of non-redundant protein database (nr, available at www.ncbi.nlm.nih.gov) search.

L'identification des protéines se fait aujourd'hui principalement par spectrométrie de masse

- Isotope profiles (muscles and otoliths)

Stable isotopes, including nitrogen and carbon can be studied particularly in ecology to estimate trophic position of an organism (Minagawa and Wada, 1984). In this study, the isotopic signatures will not be considered as signals linking the trophic structure, but as integrators of the diet, and also as indicators of metabolic activity of the animals. $\delta^{13}C$ et $\delta^{15}N$ will be measured in muscle tissues, $\delta^{13}C$ and $\delta^{18}O$ values in the otoliths (calcified inner ear fish bones). In the fish otolith, the $\delta^{18}O$ signature is strongly correlated with the temperature of the medium, while seasonal and ontogenetic variations of $\delta^{13}C$ signatures could be strongly related to the rate of metabolic activity and thus could become a very useful tool for ecophysiological studies (Kalish, 1991; Dufour et al, 2007.).

- Population genetics approach: AMPD1 gene polymorphism

The AMPD1 is a gene that encodes an enzyme that catalyzes the deamination of adenosine monophosphate (AMP) to inosine monophosphate (IMP), which allows the regulation of adenylate load (AEC), and therefore plays a key role in the management energy at the cellular level. It seemed to be a relevant candidate gene potentially undergo selection pressures in contaminated environments where the stress response of fish results in a metabolic cost therefore (Marchand et al., 2004).

The polymorphism of this gene has been explored by the HRM (High Resolution Melting Analysis) technique. This gene consists of 12 exons and 11 introns, in this study only the polymorphism of exon 1 (160 bp) was analyzed because it is the only exon that showed a strong polymorphism in natural populations.

III.4.3. Exposure experiment

Juvenile flounder (0 +) from a farm were contaminated through food with a cocktail of PAHs and PCBs designed to reflect the levels of contamination found in the Seine (C1) and 10 times these levels (C2). The samples were taken after 15 days of acclimatization (T0), 14 and 29 days of infection (T14 and T29), and after 14 days of decontamination (T43) (Figure 38).

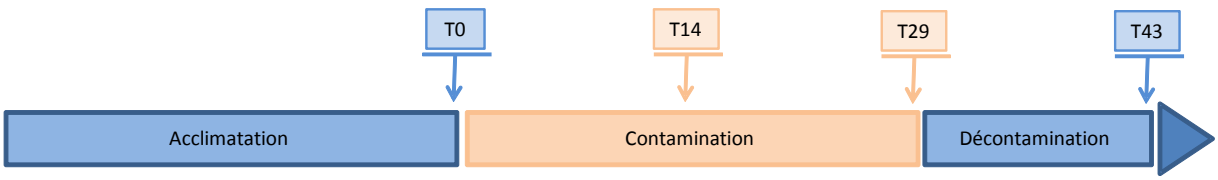


Figure 38 : Experimental design.

Contaminants were measured in the granules (PCBs and PAHs) and the carcasses of fish T29 (PCB). The results (Table 1) show a ratio of about 10 between C1 and C2 in the granules as in carcasses, indicating that the route of infection was appropriate. In addition, weight gain (SGR) is

correlated with the concentration of contaminants in the condition C1 (confirming that fish eat more is contaminated).

Cocktail at C2 level causes over-expression of genes CYP1A1 and BHMT after 29 days of infection, and this induction is maintained after 14 days of recovery. The induction of these two genes can be explained by the establishment of detoxification. In addition, 2D proteomics study showed, after 29 days of contamination at a C2 level, an increase in abundance of BHMT and SHMT, two proteins involved in the metabolism of methionine. This increase may be associated with an increase in antioxidant defenses (GPx) and suggests that BHMT and SHMT are involved in a cycle resulting in the production of glutathione.

Maintaining the over-expression of genes CYP1A1 and BHMT after the restoration phase may indicate that metabolites of PCBs or PAHs are still present in fish in sufficient quantities to induce detoxification process. To confirm this hypothesis, Veronique Loizeau conducted in February and March 2013, additional measurements of PCBs in contaminated carcasses at a C2 level to 43 days fish.

Tableau 7 : PAH & PCB in food: nominal (Targeted) and actual values (Measured).

	control with iso-octane	C1		C2	
	measured concentration (ng/g)	targeted concentration (ng/g)	measured concentration (ng/g)	targeted concentration (ng/g)	measured concentration (ng/g)
HAP :					
fluoranthene	2.3	210	189	2100	2072
pyrene	3.2	200	176	2000	1916
benzo(a)anthracene	0.7	70	49.6	700	640
chrysene	1.5	130	84.8	1300	1144
benzo(b)fluoranthene	2.3	170	116	1700	1501
benzo(k)fluoranthene	0.7	60	43.3	600	529
benzo(a)pyrene	1.3	60	42.1	600	517
indeno(1,2,3-cd)pyrene	nm	50	nm	500	514
benzo(ghi)perylene	0	50	54	500	435
∑ HAP =	12	1000	754.8	10000	9268
CB :					
CB 28	0	5	4.2	50	47.1
CB 52	0.4	25	24.3	250	252
CB 101	1.6	50	43.8	500	470
CB 149	2.1	50	42.8	500	477
CB 118	1.1	50	41.7	500	465
CB 153	3.3	100	86	1000	939
CB 105	1.4	25	20.3	250	238
CB 138	2.6	100	79.4	1000	911
CB 156	1.2	25	20.3	250	218
CB 180	1.4	50	37.9	500	449
CB 170	1.5	25	17.7	250	212
CB 194	0	5	3.5	50	42.5
CB 126	0	5	3.9	50	46.9
∑ PCB	16.6	515	425.8	5150	4767.5
total load of contaminants	28.6	1515	1180.6	15150	14035.5

After 29 days of contamination at C2, the levels of DNA damage were significantly higher (relative to control fish) and correlated with the expression of CYP1A1 levels ($R = 0.964$, $p < 0.05$). This suggests that the production of metabolites of PAHs could lead to an increase in DNA damage (Lee and Steinert, 2003). In addition, proteomics approach showed an increase in antioxidant defenses that may be associated with the presence of ROS, they may also cause DNA damage (Lee and Steinert, 2003). The absence of DNA damage after 43 days suggests that the level of metabolites and/or ROS has fallen below the limit that causes genotoxicity measurable by the comet assay.

Immunological markers are significantly affected by the pollution, but their answers are complex to analyze. C3 and TNF are induced in the presence of pollutants after 29 days at C2, while conversely, after exposure to C1 the amount of lysozyme is reduced from 14 days on. The complement and TNF are both involved in the inflammatory response. Chemical pollutants are known to cause inflammation in fish (eg Pacheco and Santos, 2002), which could explain the induction of TNF-R and C3. Meanwhile, the decrease in lysozyme expression, suggests that an immunotoxic effect is occurring. Moreover, the lysozyme is the most sensitive immune markers analyzed in this study, since it is modulated at the C1 level from 14 to 29 days.

Exposure to C1 induces no proteomic response, relative to the control condition, in addition, among the biomarkers measured, only two immunological parameters are modulated by the level of contamination C1 equivalent to that of the Seine (the lysozyme at day14 and C3 at d29 and d29). Yet several studies have observed of DNA damage or induction of CYP1A1 in fish from the Seine bay (eg Akcha, Vincent Hubert and Pfhol-Leszkowicz, 2003). This lack of response in an experimental situation can be explained by the short duration of the period of contamination (only one month) and the absence of effective substances in our cocktail. Indeed, the mixture of PAHs and PCBs whose effects are explored in the laboratory, obviously cannot simulate the complex mixture of pollutants in the Seine.

In conclusion, this experiment shows that the markers used are modulated by contaminants in our experimental situation. Although the immune system is complex, it is clearly affected by chemical stress. Moreover, the existence of a link between CYP1A1 and DNA damage was confirmed in this experiment.

The proteomic study showed that flounder exposed to complex mixtures of PAHs and PCBs experienced a deregulation of their energy and methionine metabolism. Detoxification enzymes and antioxidant defense accumulated after 29 days of contamination. Cell glutathione needs appeared to increase both for the detoxification of xenobiotics by GST and the anti-oxidant defense (GPx). The hypothesis of the involvement of BHMT and SHMT in a cycle resulting in the production of glutathione can be made (Figure 40).

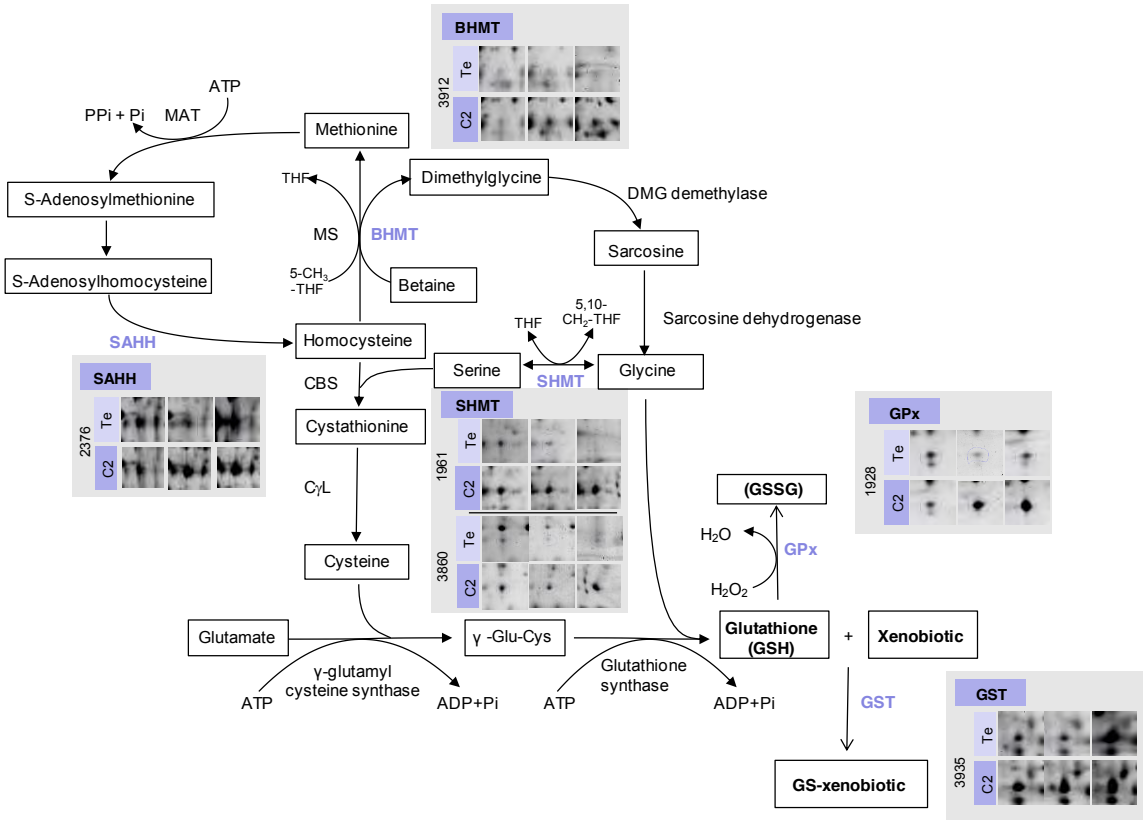


Figure 40 : Proposal for a cycle involving BHMT, SHMT, SAHH, GST and GPx. For each identified protein, the views presented for spots T29Te conditions (control animals after 29 days) vs T29C2 (C2 contaminated animals after 29 days). CBS, cystathionine beta synthase; CγL, cystathionine gamma lyase; MS, methionine synthase; BHMT, betaine homocysteine methyltransferase; SHMT, serine hydroxymethyltransferase; GST, glutathione S transferase; GPx, glutathione peroxidase.

III.4.4. In situ study

Juvenile fish (young of the year: YOY or 0 +) and juveniles of more than 1 year (1 +) were captured in three estuaries (Fig. 39): the Tamar (England), the Seine and the Canche (France). The Canche Estuary is considered in many studies as a reference site and display a low level of contamination (eg Amara et al, 2007. Amara et al, 2009.). The Seine estuary is very polluted, with a very complex mixture of pollutants (PAHs, PCBs, heavy metals, etc...) (Loizeau and Abarnou 1995; Munschy et al, 1996). Tamar has high concentrations of metals that are the result of past mining activities (Langston et al., 2003).

The results are summarised here in table 8. Existing correlations between the measured molecular parameters were calculated for the estuaries of the River Seine and the Canche in 2010 for two age classes (0 + and 1 +).

Conversely to the experimental conditions, the activity of CCO is different between sites for the 0 + cohort and is higher in the Seine activity than in the Canche. It could reflect an energy surcharge for contaminated fish, related to the mechanisms of protection/compensation.

At the detoxification process, the expression of CYP1A1 does not appear to be induced in contaminated sites. The measurement of the EROD activity may better reflect the detoxification process. However, the BHMT, which is also related to the detoxification process, is induced in the Seine for the cohort 0 +.

Figure 39 : The three Channel estuaries (England : Tamar ; France : Canche, Seine) and two supplementary estuaries (Vilaine and Mondego) sampled in this study.

As seen in experimental conditions, the expression of TNF-R and C3 genes is increased in polluted estuaries. Expression of C3 may be a less sensitive indicator of metal pollution in the Tamar, as it is induced in Seine while the TNF-R is activated Seine and Tamar. The most likely hypothesis is that the presence of pollutants causing inflammation, the complement system and TNF are involved in the inflammatory response would then be activated. Moreover, the ability of phagocytosis is diminished both for the cohort 0+ and 1+ in the Seine in 2010. This suggests that the chemical stress can have an immunosuppressive effect that could make fish less resistant to pathogens in highly polluted populations

Tableau 8 : Variations of a suite of biomakers measured in fish from the Canche, the Seine and the Tamar. Phago=phagocytosis activity, reins a.= head kidney, cyto=cytometry, activ.= enzymatic activity, ref. = control site. ++ or - - means that the value is twice more or less that of the control site.

indice mesuré		detoxification		système immunitaire			metabo.	indices de condition
		CYP1A1	BHMT	phago.	C3	TNF-R	CCO	CF
organe		foie	foie	reins a.	foie	foie	muscle	indiv.
technique		RT-PCR	RT-PCR	cyto.	RT-PCR	RT-PCR	activ.	indice
terrain - flet								
2009	Canche	ref.	ref.	n.m.	ref.	ref.	ref.	ref.
	Tamar	0	0	n.m.	0	+	0	-
	Seine	n.m.	n.m.	n.m.	n.m.	n.m.	+	-
2010	Canche 0+	0	0	ref	0	0	0	0
	Canche 1+	0	0	0	0	0	0	0
	Seine 0+	0	+	- -	+	+	++	0
	Seine 1+	0	0	- -	+	+	0	0

The observation of the profiles obtained by 2-dimensional electrophoresis (Fig. 41) showed a convergence in patterns of fish in the Tamar and the Seine over the fish of the Canche. The patterns observed in the livers of fish from the Tamar and the Seine are characterized by dysregulation of energy metabolism and may reflect an increase in energy demand (increased accumulation of MDH and FBPA). However, the β-globin, a protein involved in the transport of oxygen and accumulated in response to increased energy demand, seems instead decrease here. These results could be explained by the presence of local adaptation. Indeed, local adaptations have been described in *P. flesus* in some populations of northern Europe in living environments with contrasted salinity. Larsen et al. (2007) showed that, although the genetic differentiation is low between the different populations studied, they have different capacities including the induction of ALAS, the enzyme catalyzing the first step in heme synthesis. In addition, the GST, involved in phase II enzymes and antioxidant defenses, is accumulated in fish livers of the Seine and the Tamar. Finally, a protein from the vitellin superfamily, a membrane outer layer protein 1 (VMO-I) is significantly accumulated (with an induction factor of 6.7) in the livers of fish in the Seine while it is totally absent in fish from the Canche or the Tamar. The protein produced in the liver, is involved in vitellogenesis and could be an early marker of endocrine disruption.

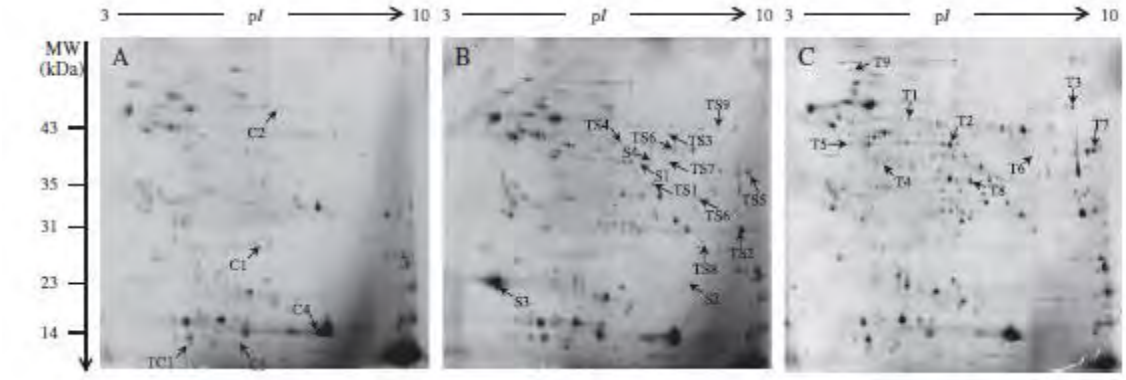


Figure 41 : 2D gels from liver proteins extracts of fish from (A) the Canche, (B) the Seine and (C) the Tamar. Proteins differencially expressed are indicated with number. Each gel is a representative of 4 independant replicates.

The study of otolith microstructure on transverse sections show that these fish are subjected to many stresses throughout their life of larvae and juveniles which is characterized by significant morphological changes during their ontogenetic development. For 2009, the estimated growth rate of fish from the Canche (3.1 ± 0.8 mm / d, 12 individuals) was significantly lower than those observed for fish from the Seine (3.8 ± 1.1 mm/d, n= 23) and Vilaine (3.8 ± 1.1mm/d n= 23). The fish of the Canche, living in the lowest polluted area, are both smaller and have the lowest daily growth rate. This a priori surprising and may be explained by the fact that factors other than the intensity of chemical stress the so-called "confounding factors of the environment" can affect growth in natural environments

The compositions of stable isotopes of carbon and nitrogen were acquired on fish muscle from estuaries during 2009 and 2010. They are potentially relevant indicators of the rate of metabolic activity. Different signatures were observed between the three estuaries (Fig. 42). The δ13C signatures and δ15N of fish in the Seine are relatively stable in 2009, then dispersed in 2010. Samples from the Canche 2009 have a very homogeneous signature for the two tracers with the highest δ13C values of the three estuaries. We can hypothesize that individuals captured in 2010 vs. 2009 were of more diverse geographical origins. In Canche the lowest δ13C values can be explained by the contributions of continental organic matter having a weak signature. The fish of the Vilaine clearly distinguished by high δ15N values and a very high stability of their signature over the years (Fig. 42) suggesting that they are at a higher trophic level than those in other estuaries. This can be linked either to different carbon sources, or to differences in metabolic rate between these two estuaries.

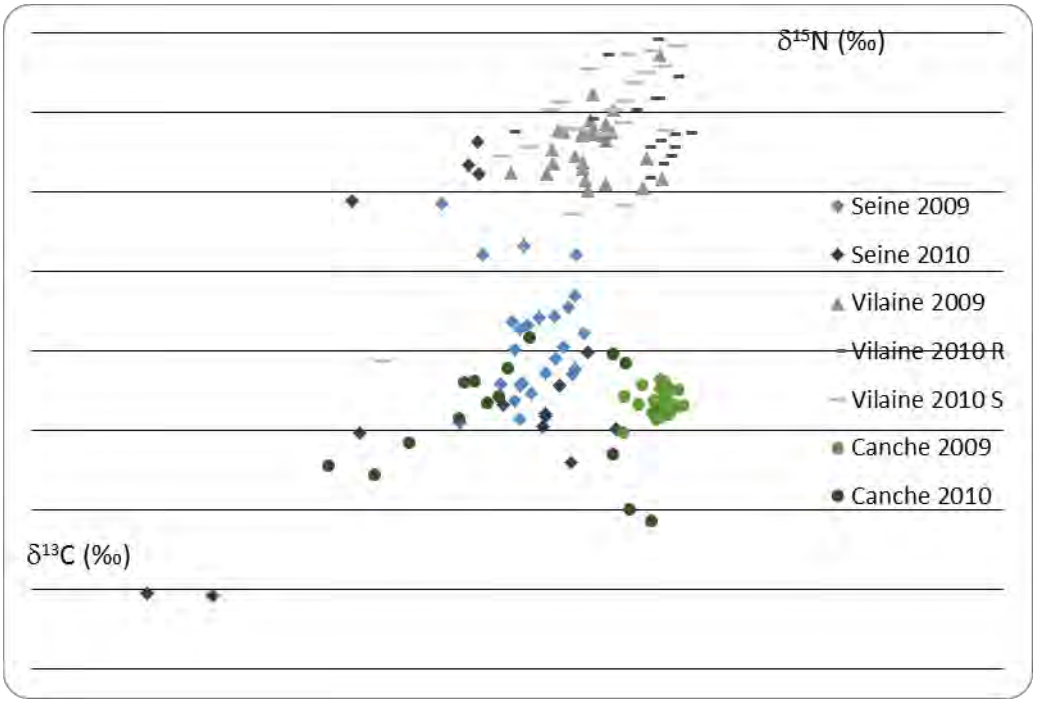


Figure 42 : Isotopic signature of flounder muscles from the Seine, Vilaine and Canche in 2009 and 2010. R= resistant, S= sensitive to hypoxia.

In otoliths, stable isotopic compositions of oxygen and carbon were measured (Fig. 43). There is a strong differentiation between samples Canche (low $\delta^{13}C$ and $\delta^{18}O$) and Vilaine (high $\delta^{13}C$ and $\delta^{18}O$) that can be explained by life history traits and habitat occupancy completely different for these two populations (salty environment in the Vilaine vs. freshwater for the Canche). Variations in $\delta^{18}O$ are strongly related to the temperature of the environment, with higher temperatures in the Canche (more upstream habitat) than in Vilaine (more downstream habitat). Moreover, the otolith $\delta^{13}C$ signatures allow to significantly differentiate the three populations, much better than for muscle signatures. This may indicate that different carbon sources and/or metabolic rates are different between these estuaries. More generally, the strong negative linear relationship between the detected metabolic activity rate of a fish and the $\delta^{13}C$ value obtained in its otoliths (Dufour et al., 2007) strongly suggest an increase in the average temperature of the environment correlated with an increase in the metabolic rate of the fish in the sense: Vilaine, Seine and Canche.

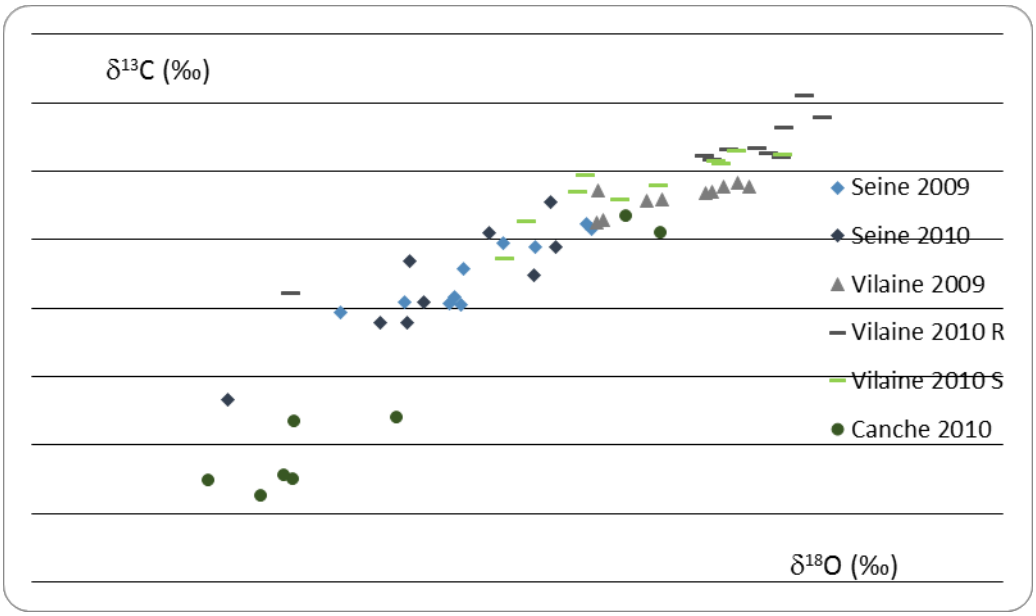


Figure 43 : : Isotopic signature of flounder otoliths from the Seine, Vilaine and Canche in 2009 and 2010. R= resistant, S= sensitive to hypoxia.

The microchemical analysis and particularly the profiles of Barium and Strontium along the otolith, from birth to the catch, says the strategies of occupation of the differential space between estuaries. Indeed a high Sr concentrations indicate a marine origin and a lower value, an estuarine phase. Opposite, high concentrations of barium are connected to continental inputs and low to marine phase.

We observed that fish from the Canche and the Vilaine were born in marine environments (high Sr), unlike the Seine where births are estuarine, and a passage in desalinated water (low Sr) which occurs after metamorphosis, materialized by a red line (fig. 44). Then the flounder in Canche have a life mainly located in desalinated water, for both the 0+ and 1+. Of the 19 fish analyzed, 17 showed profiles with freshwater signatures throughout their lives. Juveniles from the Vilaine have an essentially marine life, with a phase marked with a slightly lower level of Sr, indicating a period in the upstream part of the estuary which is limited by a dam located 12 km from the mouth. Juveniles of the Seine spend a significant part of their life cycle in much desalinated water, then migrate to more saline waters. These strategies of space occupation by juvenile flounder in estuaries, could be put in relation with the observed growth rate in the Seine-and the Vilaine vs. the Canche. The continued presence in some salt water in the Canche system could explain the relatively low growth rate.

Canche Vilaine Seine

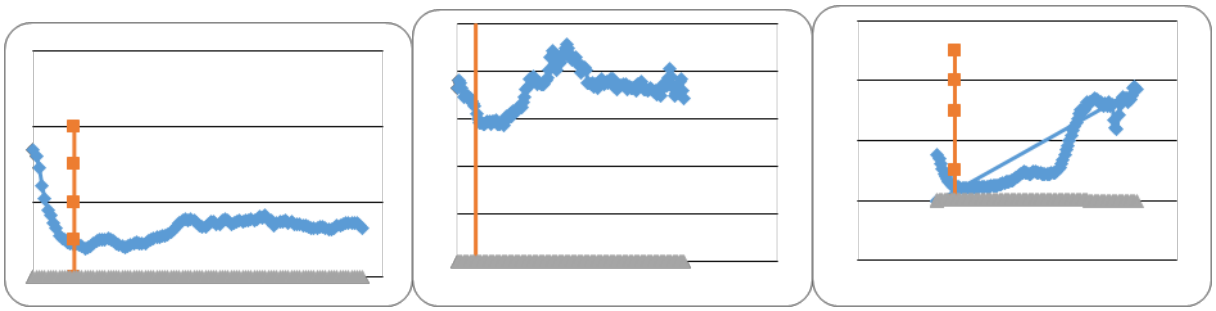


Figure 44 : Concentrations en Strontium en bleu (échelle de gauche en ppm), et en Baryum en vert (échelle de droite en ppm), le long d'un transect en µm, du nucleus (naissance) à la capture, pour des poisson issus de Canche, Vilaine et Seine en 2009. La ligne droite rouge indique la zone de métamorphose.

Hypoxic challenges were conducted in the laboratory on flounder from Vilaine in 2010 (subject to a chronic hypoxia in their estuary in summer) and have led to the identification of two groups of fish, S "sensitive" or R "resistant" to hypoxia. The comparison of the isotopic signatures of the muscles between R and S in 2010 showed no difference in the $\delta^{15}N$, but differences close to the limit of significance were obtained for $\delta\ ^{13}C$ ($p = 0.054$) (Fig. 42). These results are confirmed by the analysis of stable isotopes in otoliths that incorporate the entire life of fish. A significant difference in the $\delta\ ^{18}O$ and $\delta\ ^{13}C$ between R and S is then observed (Fig. 43), the most resistant having higher $\delta\ ^{18}O$ and $\delta\ ^{13}C$ levels. These analyzes indicate that the trophic level is probably the same for both R and S, but the carbon sources diverge and/or the metabolic rates are different.

Profiles of trace elements on flounder from the Vilaine sampled in 2010 indicate two types of occupation strategies of the estuary, which not detected in 2009 (Fig. 44). Some fish are primarily marine, while others are living in freshwater after metamorphosis. 3/8 of the fish are "marine" in the sensitive group (high concentrations of Sr and low Ba), and 5/10 in the resistant group. These results suggest that the changes in $\delta\ ^{13}C$ between R and S might be less associated with different sources of carbon than with the fish metabolic rate, hypoxia-resistant fish having a lower metabolic rate than the sensitive fish.

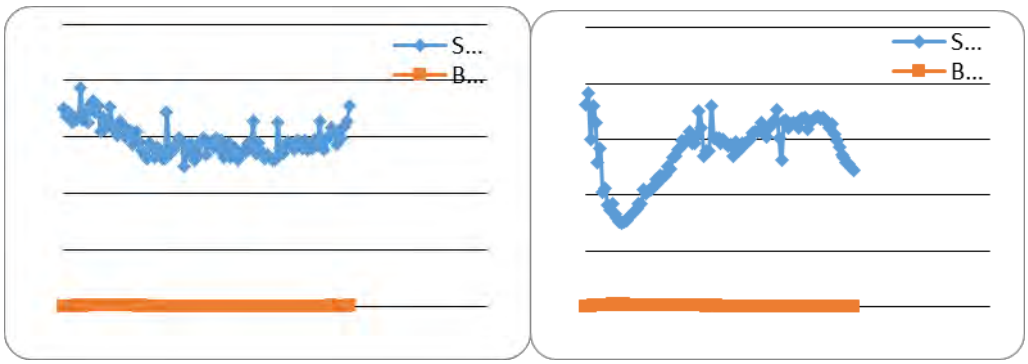


Figure 45 : Two types of Sr and Ba profiles of the otolithes of flounder from the Vilaine estuary. Concentration are quantified in different parts of the otholith from the nucleus to the edge. The left

panel is showing a typically “marine” fish, the right profile is representative of fish having a freshwater phase.

Variability AMP-deaminase gene isoform 1 (involved in energy metabolism) was studied on four flounder populations from the Tamar the Canche the Seine, the Vilaine and the Mondego estuary. 40 juvenile flounder were sampled in each estuary and genotyped.

The genetic diversity and the observed heterozygosity (Ho) allowed to highlight the following pattern:

Ho Seine (0,480)> Ho Tamar (0.413)> Ho Mondego (0.358)> Ho Vilaine (0,301)> Ho Canche (0.29)

A positive correlation between the level of water pollution and genetic diversity might be present. We hypothesize that the selection pressure exerted by chemical contaminants directly or indirectly affect the AMPD gene expression in natural populations in estuaries, the most heterozygous genotypes potentially would present a greater ability to "resistance" against the chemical stress.

The inter-populational genetic diversity was analyzed by MDS (multidimensional scaling) it highlights a significant genetic differentiation between the most polluted estuaries Seine & Tamar vs. the moderately or slightly contaminated estuaries: Vilaine Mondego Canche (Fig. 46).

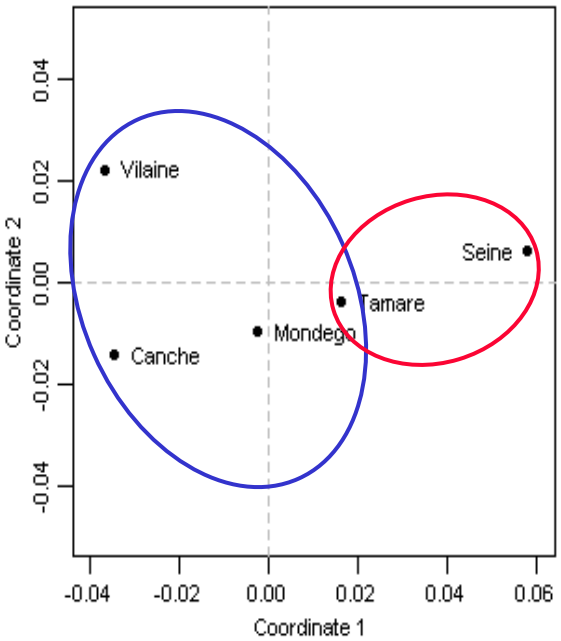


Figure 46. Multidimensional scaling of the flounder genetic diversity in five estuaries.

III.4.4. Perspectives in terms of tools for research and monitoring programs of natural environments affected by chemical stress

Overall it is clear from this work that biomarkers of the immune system are particularly relevant in ecotoxicology, under controlled conditions, or in natural environments. In particular the activity of lysozyme is one of the most sensitive markers to chemical stress. This activity can be measured in the blood but also potentially in the mucus. Overexpression of the C3 and TNF-R genes is also confirmed in experiments and in situ studies. The phagocytosis capacity is clearly affected by pollutants in natural population's environments. Overall it can be assumed that changes in immune capacity of fish exposed to chemical stress can potentially: (1) increase their susceptibility to pathogens, (2) lead to a loss of fitness in natural populations. Alterations in the immune system will be deepened in the future, by bacterial or viral challenges implemented in polluted populations vs. the populations of "reference systems".

Following the work on proteomics, the deregulation of the energy metabolism, induction of antioxidant and detoxification mechanisms were observed. These mechanisms have been extensively studied and their potential as biomarkers evaluated. Conversely, the potential as biomarkers of VMO-I and enzymes involved in the methionine cycle, including BHMT is so far little studied but showed high potentials.

Another original approach of this study was to consider the isotopic signatures (on muscle and otolith) and microchemistry (on otolith) as these signatures are extensively used in ecology, but still little explored in the context of ecotoxicology. Interannual variation of the isotopic signatures observed in fish sampled in a given estuary, has enabled us to show that fish might have lived in different part of the estuary. This dispersion could lead to a sharp increase in interindividual variability of biomarkers, as the bioavailability of pollutants can be strongly linked to local hydroclimatic factors (salinity, temperature, organic matter load, etc.). Isotopic signatures in the otolith have also proved very relevant as proxies of environmental temperature ($\delta\ ^{18}O$) and the metabolic activity of fish ($\delta\ ^{13}C$).

Microchemical markers (Sr, Ba) have been very informative to detect the likely effects of confounding factors in the natural environment, not directly related to chemical stress. Thus, the low daily growth rate of fish in the "clean" estuaries is most likely associated with the development of juvenile fish in the much desalinated part of the system, instead of the more marine part of the polluted estuaries. The use of these markers could be developed in ecotoxicology to better understand the complex responses of fish in natural systems such as estuaries.

Finally, the integration of population genetics in ecotoxicology can become very relevant: 1) to study the correlation between the genetic variability of different candidate genes and the level of pollution in natural systems; and 2) to detect differences in physiological performance between fish with certain alleles or genotypes in polluted environments, and thus to explore the mechanistic basis of resistance to pollutants (Wirgin et al, 2011; Marchand et al, 2013).

IV. *IN SITU* MONITORING IN THE MANCHE REGION

IV.1. Introduction

For about thirty years, the research efforts in the field of Ecotoxicology led to the development and characterization, of several biological indicators such as biomarkers and bioassays that may be used for aquatic monitoring. If the recent Marine Strategy Framework Directive (Directive 2008/56/EC on the definition of good environmental status in the marine waters) proposes the use of biomarkers in key functions or to study mechanisms of action of specific pollutants, it does not hold true for inland water bodies for which the Water Framework Directive (2000/60/EC) includes only monitoring programs using chemical and biocenotic approaches. Thus, the biological tools are not used for regulatory purposes in inland water bodies although various studies highlighted the potential and value of these measures in addition to or instead of conventional approaches.

The present work aimed at highlighting the interest of a battery of biomarkers measured in the INTERREG France-England Channel area. The aim is to provide, using a series of biological responses and analytical tools from multiple levels of biological complexity, an assessment of the quality of aquatic systems. In this context, we focused on the contamination of aquatic ecosystems and its effects on organisms induced by endocrine disruptors (estrogen, androgen and anti-androgens), genotoxic and immunotoxic compounds. To meet this objective, several sub-actions have been taken:

1. to develop tools and methods for biomarker studies including validation and quality assurance measures;
2. to study links between exposure, biomarkers and physiological parameters;
3. to develop a method to sum-up several biomarkers measurements into a single, simple and easy to understand value that could be used by environmental managers;
4. to map shellfish and wild fish biomarkers responses in the study area.

IV.2. Reference gene selection for quantitative real-time PCR in mussel, Mytilus edulis, during gametogenesis and exogenous estrogen exposure

To date, an ideal universal reference gene that is stable under different experimental circumstances has not been described. Accordingly, the choice of a reference gene has to be determined based on the stable expression in a particular tissue or under the given experimental condition. Furthermore, the use of a single reference gene can lead to errors and it is advisable to use multiple reference genes for calculation of a reliable normalization factor. Several suitable reference genes in human tissues have been identified and validated; yet studies comparing the stability of reference gene expression in non-vertebrate species including molluscs are relatively few. Several mathematical algorithms are available to assess the suitability of candidate reference genes as normalizing genes in qPCR experiments. GeNorm and NormFinder are freely available Visual Basic applications for Microsoft Excel tools for evaluation of stability reference genes.

As part of our investigations regarding variation of expression in key genes involved in endocrine disruption, we compared the stability of six reference genes (*beta actin*, *alpha tubulin*, *18S ribosomal RNA*, *28S ribosomal RNA*, *elongation factor-1 alpha* and the *DEAD-box RNA helicase*) in mussels, *M. edulis*, at different stages of gametogenesis and following experimental exposure to a model estrogen. GeNorm and NormFinder softwares were employed and the effect of reference gene selection on the interpretation of qPCR-generated data assessed. The most stable reference genes for

mussels (in these circumstances), *EF1* and *EF1*/*TUB* in combination, revealed a significant decrease of *ER* mRNA relative levels in mature mussels compared with early developing mussels after estradiol exposure. We demonstrate that the experimental results are highly dependent on the reference gene chosen and that statistically significant contrasting differences between sample groups are present or absent depending on the reference gene employed.

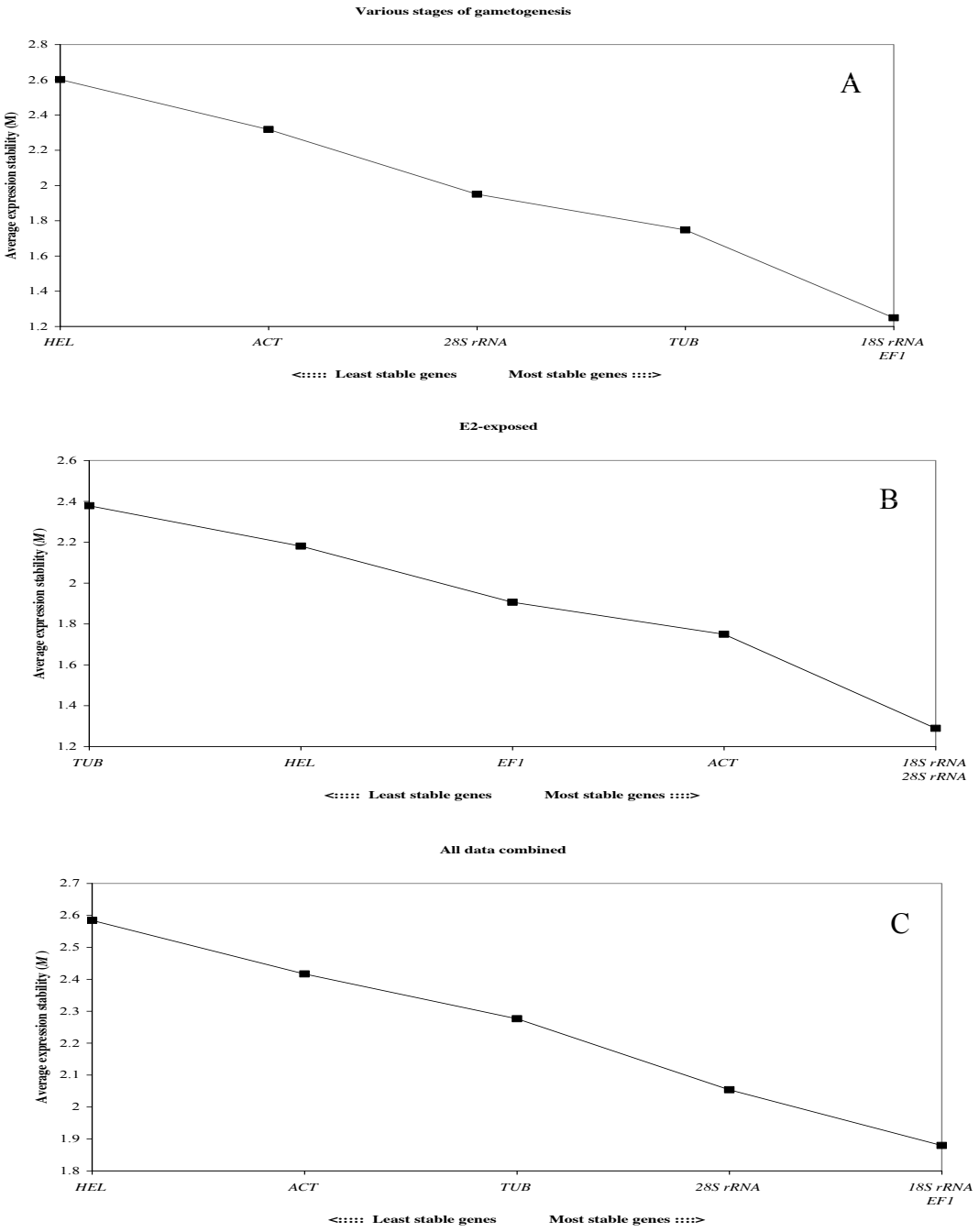


Figure 47: Normalized relative expression levels of *ER* in developing and mature mussels (n=8). Mean data are plotted ± standard error of the mean (S.E.M.). * indicates significant differences of $p < 0.05$ when compared to developing mussels, ** represents significant differences of $p < 0.01$.

In conclusion, preliminary evaluation of gene stability is required prior to every study to obtain the most reliable results.

IV.3. Identification of a new sentinel species: the bullhead (*Cottus sp.*)

The longitudinal distribution of fish in the rivers and within watersheds makes it impossible to use a single sentinel species for the implementation of a multi-biomarker approach. In addition, using recognized sentinels species such as roach (*Rutilus rutilus*), gudgeon (*Gobio gobio*), chub (*Leuciscus cephalus*) or stickleback (*Gasterosteus aculeatus*) does not guarantee an optimal coverage monitoring networks. To fill this gap, one of the objectives of this action was to characterize the potential of a new sentinel species: the bullhead, to measure a set of biomarkers. Indeed, bullhead is a benthic fish widely distributed in European inland aquatic ecosystems of varying levels of contamination. Its small size and good knowledge of its biology makes it easy to implement the characterization of biomarkers in controlled conditions. In addition, its use in the context of biomonitoring has already been shown to be relevant (Bucher and Hofer, 1993, Bucher et al., 1992, Hofer, 1996). We thus developed a multi-biomarker approach in bullhead and characterized the biomarkers.

This work began with the study of the reproduction of wild populations of bullhead. Indeed, as these fish show a complex mode of reproduction, it was a pre-requisite before considering the use of this species as an indicator of contamination. This first step confirmed the existence of two reproductive strategies in bullhead (annual or seasonal) inherent in our study area and highlighted the involved environmental factors. In addition, this study confirmed the enlarged kidney in bullhead males during the breeding season and led to the development of a histological method to quantify this androgen-regulated phenomenon (the thickness of the renal epithelium, see part 2 of the report).

Preliminary work aimed at optimizing and validating the biochemical assays. Several colorimetric or fluorimetric assays of selected biomarkers previously developed in the laboratory on other fish models such as the rainbow trout, *Oncorhynchus mykiss* (Ait Aissa et al ., 2003), *sea bass*, *Dicentrarchus labrax* (Deviller et al., 2005), three-spined stickleback, *Gasterosteus aculeatus* (Sanchez et al., 2007) and chub, *Leuciscus cephalus* (Hinfray et al., 2010) were applied to the bullhead.

To validate the biomarkers several parameters were defined:

- The limits of detection (LOD) and quantification (LOQ) are respectively defined as the smallest amount of substance or enzymatic activity of the test sample can be detected, and the smallest amount of substance or enzyme activity can be assayed by the method. These limits are determined from the measured reaction rates for the "controls" according to the following relationships: LD = mcontrol + 3SD LOQ = 10SD + mcontrol

where mcontrol corresponds to the average value obtained for “controls" and the associated standard deviation SD.

- The zone of linearity of the calibration curve which is defined by the highest concentration tested, which allows to obtain a linear relationship between the measured signal and the concentration or activity of the standard.

- The protein load or the optimal dilution of the sample to perform the assay under conditions where the signal obtained is proportional to the sample dilution. This parameter is closely related to the nature of the sample and should be redefined for each species but also for each type of organ used.

A sandwich ELISA assay was specifically developed to quantify VTG in bullhead plasma. For that purpose, VTG was purified from E2-induced bullheads according to Brion et al. (Brion et al. 2000). Figure 50 shows the dilution curves obtained by serially diluting plasma of controls male and female and estrogenized male and female bullheads. A very good parallelism was observed between

the dilution curves and the bullhead –VTG standard curve. In this figure it is also shows the dilution curves obtained by serially diluting plasma, muscle or kiney homogenates of controls male and female bullheads, zebrafish, gudgeon and three spined stickleback. No VTG is assayed in plasma from male and female stickleback, gudgeon and male zebrafish at any dilution whearas plasma from female zebrafish showed a limited response. Considering the other tissues used (muscle and kidneys), only kidney homogenates from male and female bullhead showed a limited response. Table 2 shows the precision of bullhead-Vtg ELISA method (intra- and interassay variation) and present the detection and quantification limits. The assay is caracterized by a low detection and quantification limits (8.2 an 13.9 ng/mL respectively) and presents a good precision. The coefficients of variation for the repetetability (CV inta-assay) are 9.2 ; 15.4 and 17.3 respectively for the EC50, EC20 and EC10 and the coefficients of variation for the reproductibility (CV interassay) are 33.9 ; 43.6 and 32.6 respectively for the EC 50, EC20 and EC10.

Table 9: Characteristics of the bullhead vitellogenin (Bullhead-Vtg) enzyme-linked immunosorbent assay. CV means coefficient of variation.

Parameters	EC50	EC20	EC10
Vtg concentration (ng/ml)	28.9	13.9	8.2
CV intra-assay (%)	9.2	15.4	17.3
CV inter-assay (%)	33.9	43.6	32.6

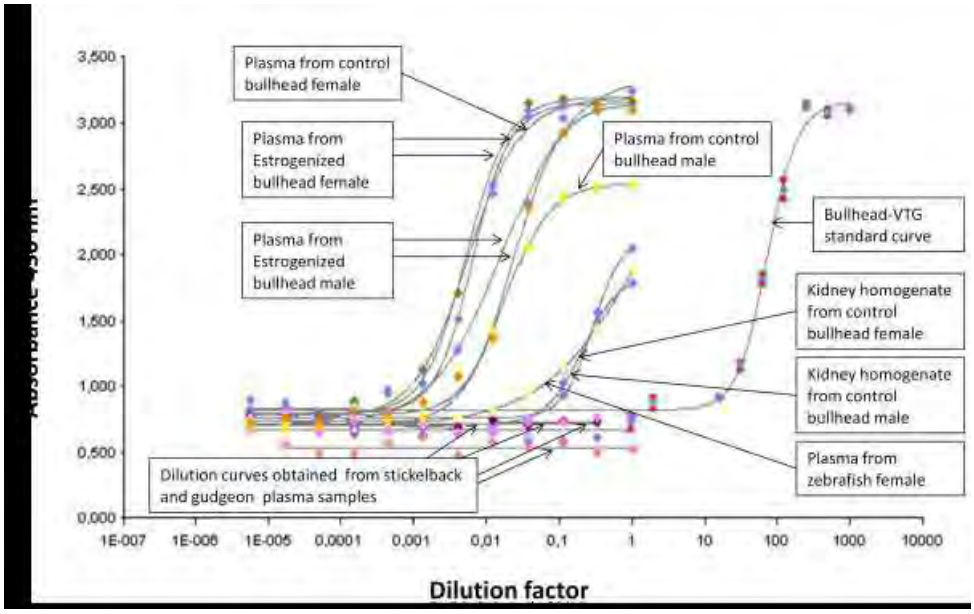


Figure 50: Dose-responses curves obtained by serial dilution of bullhead plasmas samples from male and female fish injected with 0.5 µg EE2/L, controls bullheads, male and female gudgeon, stickelback and zebrafish or from unexposed bullhead kidney extracts.

In conclusion, this work demonstrates the potential of the bullhead as a sentinel species to measure a set of biomarkers including an indicator of exposure to androgens (see Part 2 of this report). However, further experiments showed that the reproduction of this species is complex and it is necessary to take into account the hydrological regime of rivers in the interpretation of results, especially because of the existing interactions with the reproductive system. Thus, further work should be carried out to better characterize the response of these indicators and interference with biotic and abiotic factors. In addition, the recent use of monitoring data produced by fish populations ONEMA shows that the potential of other species could be investigated in connection with their wide distribution in the river systems, it is for example the case of the minnow.

IV.4. Use of biochemical and physiological biomarkers to evaluate the effects of chemical contaminants on juvenile marine fish

Attempting to relate biomarker responses of individual organisms to higher levels of biological organisation, both in the laboratory and *in situ,* offers considerable potential for improving the ecological relevance of ecotoxicological test procedures (Depledge et al., 1995). Because many toxic effects initially occur at the subcellular level, there has been an increasing use of biochemical biomarkers that help to determine causative agents responsible for altered cellular function (Schlenk et al., 1996). Among the biochemical biomarkers described in relating literature, phase I and phase II biotransformation parameters such as EROD (Ethoxyresorufin-O-deethylase) and GST (Glutathion-S-transferase) activities are currently used in environmental risk assessment (Sanchez et al., 2008). Biotransformation of chemicals is a requisite for detoxification and excretion (Gravato and Santos, 2003). Antioxidant enzymes are also commonly used to understand the associated toxic-mechanisms of xenobiotics (Sanchez et al., 2005; Oliveira et al., 2008). Many pollutants exert their effects through redoxcycling, resulting in the production of reactive oxygen species (ROS). The role of antioxidant systems is to protect the cells from this oxidative stress. Thus, measurement of components of the antioxidant defence system may be helpful to determine organism exposure to pollutant (Bilbao et al., 2010). However, although the role of biochemical biomarkers as early warning tools is recognised, it is difficult to understand their significance at higher levels of biological organisation. Indeed, in spite of their rapid responsiveness and sensitivity to contaminant exposure, biochemical biomarkers have questionable ecological relevance, as a result of being endpoints at a low level of biological organisation (Castro, 2004).

Physiological responses to chemical contaminants have often been ignored by ecotoxicologists, because they are regarded as being too generalized and too difficult to measure routinely (Depledge et al., 1995). However, evidence is now emerging that there may be some advantages in identifying integrated responses to the sum of the stresses imposed by pollutants and natural environmental factors. Juvenile fish physiological biomarkers, as growth or lipid storage, may provide the key to integrating various biochemical and cellular responses in an organism with impaired fitness; because these processes must be functional for juvenile fish to survive and so contribute to the population's renewability. Indeed, a commonly observed sublethal response of organisms exposed to chemical contaminants chronically is a change in their energy allocation (Rowe, 2003). In such a way, maintenance costs associated with combating chemical toxicants would be expected to ultimately reduce growth and energy status, and so a general decrease in juvenile fish condition.

It has long been suggested that biochemical biomarkers should be used in conjunction with measurements of fitness (Depledge et al., 1995). However, few studies have demonstrated correlative relationships between biochemical biomarkers responses and reduced fitness of aquatic organisms exposed to toxicants (Depledge et al., 1995; Lesser et al., 2001; Fonseca et al., 2009). Therefore, in the context of the DIESE program, we have proposed to develop a multifaceted approach to improve our ability to use biomarker responses to predict ecological consequences of toxicant exposure. This approach was realised by field and laboratory experiments with juvenile fish submitted to environmental concentrations of chemical contaminants observed during accidental and chronic pollution.

The first objective of this work was to analyse the ability of growth and condition indices to reflect chemical contaminants damages on juvenile fish health (Gibson, 2005; Selleslagh et al., 2009, Beck et al., 2001), and so their potentiality as biomarkers in ecotoxicology. The second objective was to study the relevance of biochemical biomarkers by analysis of their relationships with biomarkers reflecting fish health status. The last objective was to compare responses observed in field and laboratory conditions. Laboratory experiments were realised in a context of acute pollution by exposure of juvenile sea bass (*Dicentrarchus labrax*) to petroleum and then in a context of chronic pollution by exposure of turbot (*Scophthalmus* maximus) to harbour and estuarine sediments (Fig. 51). Field experiments were realised by caging juvenile sea bass and turbot in the same harbour than previously and by analysing flounder (*Platichthys flesus*) from four European estuaries more or less impacted by human activities (Fig. 52).

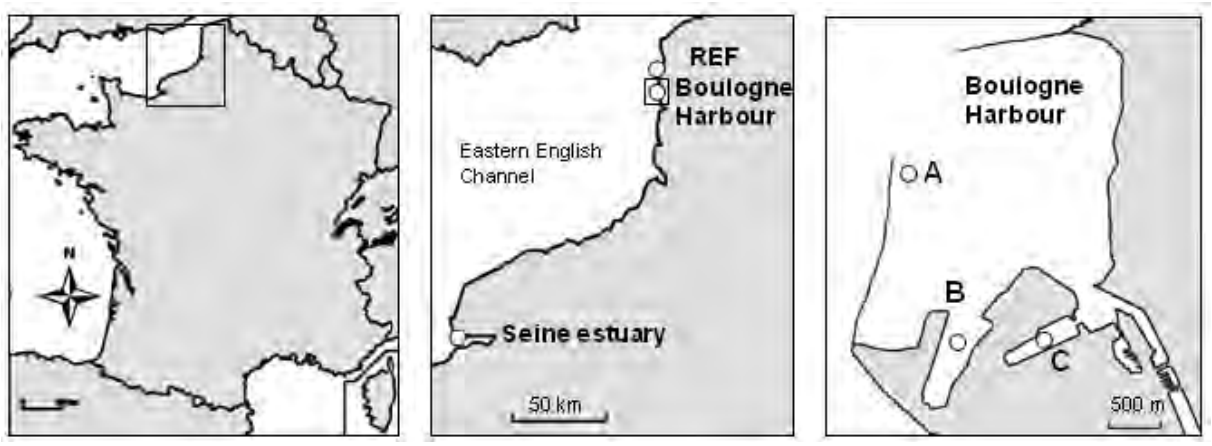


Figure 51: Map of the three sediment sampling sites (Reference, Seine Estuary and port of Boulogne sur Mer) and of the three sites within the port (A, B and C).

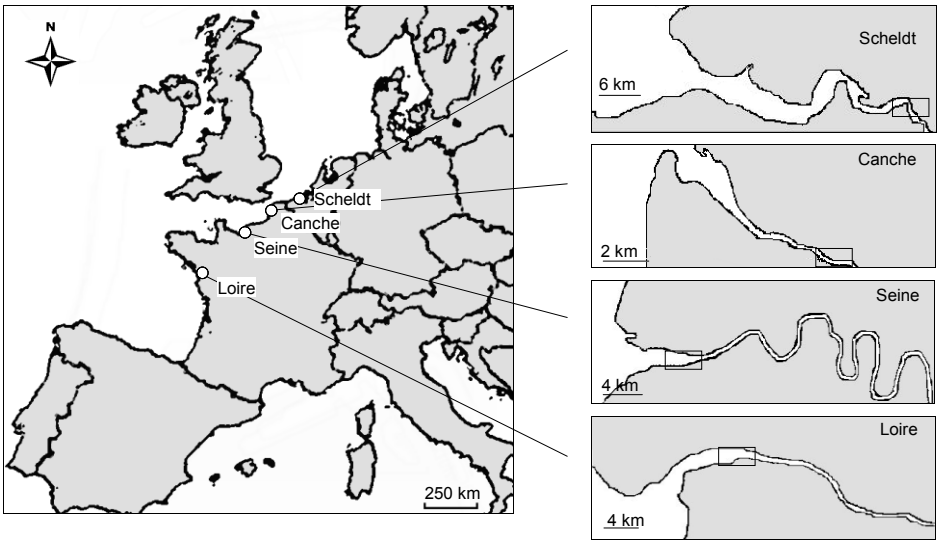


Figure.52: Map of the four estuaries (Scheldt, Canche, Seine and Loire) in which juvenile flounder were studied. The rectangles are showing the sampling areas in each of the estuaries.

Chemical measurements of body burdens are generally used as indicator of exposure to pollutant and bioavailability of contaminants. However, the results of this study showed that this is not true for PAHs as they are rapidly metabolized by adult fish (Budzinski et al. 2004) and, according to our data, even by juvenile fish. Thus dosage of PAHs in fish would not be an appropriate method to determine the levels of exposure of fish as a result of oil contamination (Collier et al., 1996). Conversely, the dosage of metals appears to reflect the levels of exposure of juveniles.

Several studies have shown that crude oil and its components inhibit the growth of fish in a number of species (Al-Yakoob et al., 1996), (Moles and Norcross, 1998), (Saborido-Rey et al., 2007). Little is known about the effects of short acute exposure to petroleum; that is why we have chosen to impose a growth period in clean seawater after this acute exposure. A significant decrease in some physiological biomarkers was observed 28 and 26 days following both exposure times. A decrease of specific growth rate in length and RNA:DNA ratio was observed after 48 h of exposure (Fig. 53). It therefore appears as though these two parameters are sensitive to oil exposure. The Fulton's K condition index, was less sensitive since it decreased only after 96 h of exposure, and lipid index showed no significant difference. The present study shows that growth and condition indices can prove useful in assessing fish health status following an oil spill.

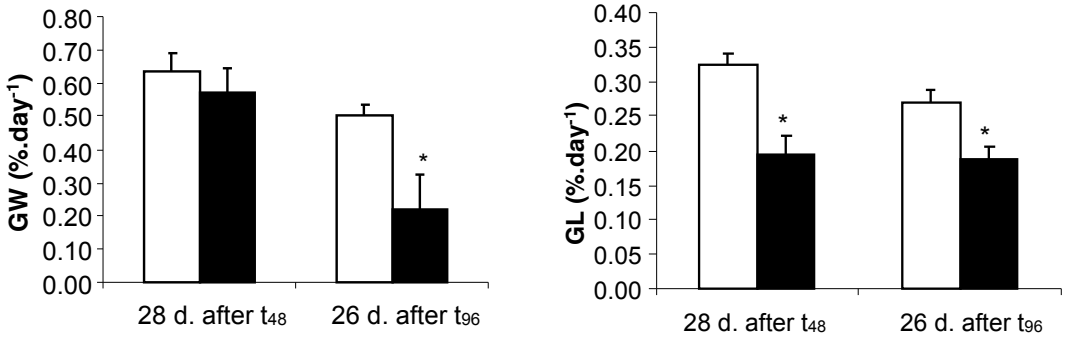


Figure 53 : Comparison of specific growth rate in length (GL) and in weight (GW) (mean ± SE), in control sea bass (CTRL) and petroleum-exposed sea bass (OIL) for 48 h and 96 h of exposure and followed by 28 and 26 days in clean sea water.

In a second laboratory study, turbot were exposed to the harbour and estuarine sediment. Turbots presented significant decrease of the specific growth rates in weight and in length with increasing level of chemical contaminants in sediments. A decrease of the RNA:DNA ratio was observed in the four contaminated conditions compared to the reference one, which confirmed the decrease of turbot growth and energetic status under chemical contamination (Fig. 54). This biochemical index has been shown to be a useful and reliable indicator of the nutritional status and growth of larval and juvenile fish, and has been widely applied to laboratory−reared, as well as wild fish (Clemmesen, 1988; Buckley et al., 1999The TAG:ST ratio was correlated with the specific growth rates and Fulton's K condition index. The decrease in the lipid index of turbot exposed to the four contaminated sediments showed that the juvenile fish appeared to have had depleted energy reserves. Lipid depletion has been identified as a general metabolic response to stress (Claireaux et al., 2004). Xenobiotic detoxification and regulation involves both passive and active mechanisms, and therefore requires energy (Alquezar et al., 2006), whereas lipids are used for energetic mechanisms associated with growth and survival.

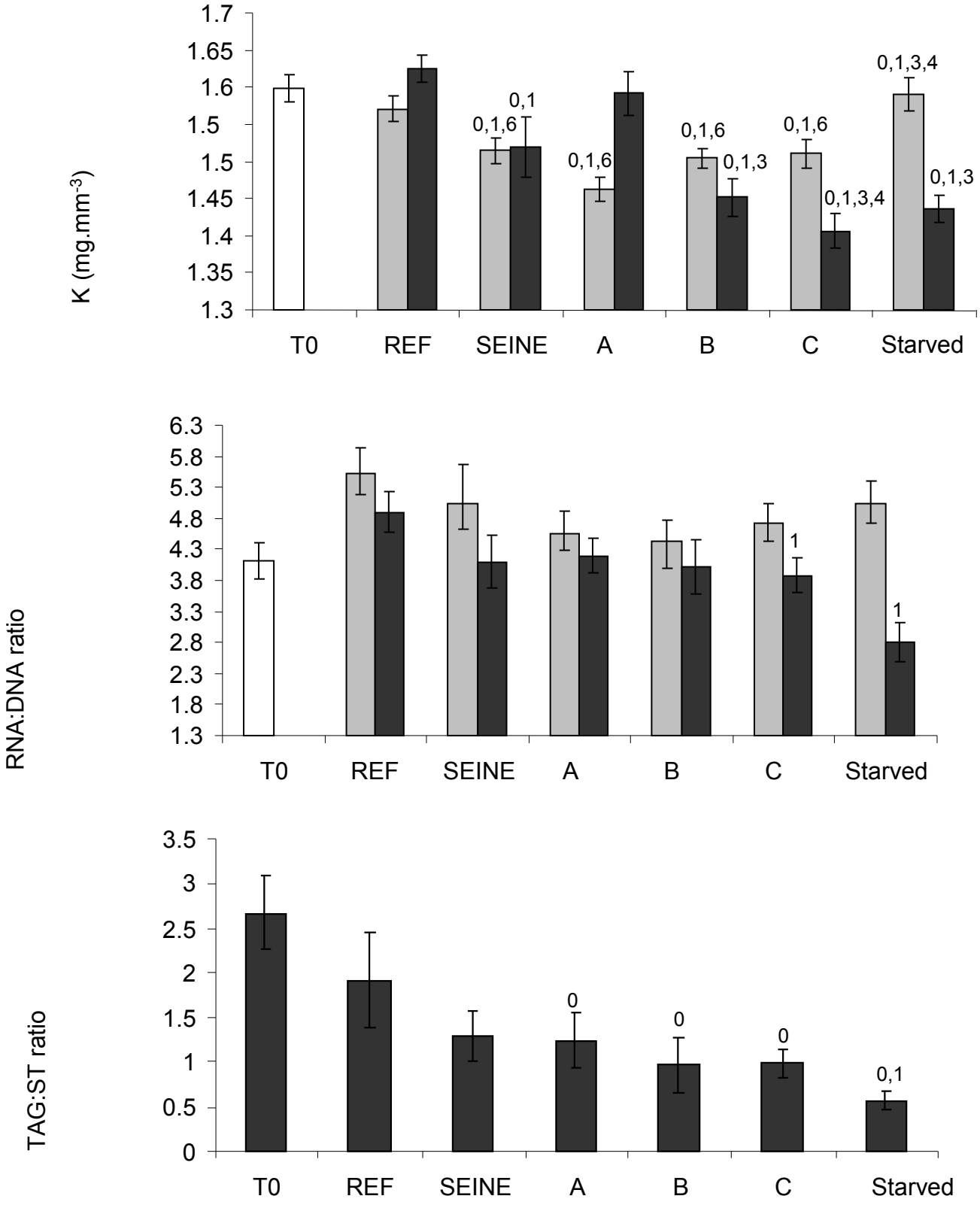


Figure 54: Fulton's K condition index, RNA:DNA ratio and lipid index (TAG:ST ratio) of turbot exposed to the five sediments (Ref, Seine, A, B and C; see maps Fig. XX and XX) during 7 (▒) and 21 (■) days (mean ± SE). n=15. (^{0}), (^{1}), (^{2}), (^{3}), (^{4}), (^{5}), (^{6}) represent significant difference ($p < 0.05$) compared to "t_0", Seine, A, B, C and starved fish, respectively.

To extrapolate these results obtained in laboratory condition on the potentiality of the use of growth and condition indices as biomarkers, field experiments were realised to analyse the role of abiotic and biotic factors occurring in environment on their responses. In first step, juvenile sea bass and turbot were caged in the Boulogne sur Mer harbour. Juvenile sea bass physiological performance showed significantly higher growth rates, RNA:DNA ratio and Fulton's K condition index in the least contaminated station (Tab. 10). The growth in weight and the condition index of the turbot caged in the less contaminated station were also higher. Similarly, for both species, lipid storage index based on the ratio of the quantity of triacylglycerols to sterols (TAG:ST), was significantly higher in the less contaminated station.

Table 10: Specific growth rate in length (GL) and in weight (GW), Fulton's K index, RNA:DNA ratio and TAG:ST ratio (mean ± SD) of juvenile sea bass and turbot caged at stations A and B (t_0: reference). For each species, ([1]) and ([2]) represent the significant difference ($p < 0.05$) compared to "t_0" and "station A" respectively..

Biological parameters	Sea bass		Turbot	
	A	B	A	B
GL	0.46 ± 0.13	0.10 ± 0.26[2]	0.04 ± 0.07	0.03 ± 0.09
GW	1.50 ± 0.29	-0.21 ± 0.34[2]	-0.19 ± 0.23	-0.39 ± 0.23[2]
K	1.03 ± 0.10	0.85 ± 0.11[1,2]	1.41 ± 0.15[1]	1.32 ± 0.06[1,2]
RNA:DNA	3.29 ± 1.11	1.71 ± 0.57[1,2]	3.39 ± 1.29[1]	3.26 ± 1.60[1]
TAG:ST	0.73 ± 0.60	0.22 ± 0.13[1,2]	0.45 ± 0.35[1]	0.19 ± 0.14[1,2]

These results of growth and condition indices of the fish caged in the harbour could be compared to the previous study in which juvenile turbot were exposed to sediments in the harbour of Boulogne Sur Mer. The growth in length and in weight measured in the turbot exposed to sediment B were similar to those found in the caged fish. However, condition indices (K index, RNA:DNA and TAG:ST ratios) were found lower in the caged fish. This comparative result suggests that environmental factors other than sediment associated contaminants, could have influenced the condition of the fish. In particular, in the laboratory study, a daily water change was performed to avoid a decrease in seawater quality. However, this renewed seawater could have decreased the transfer of chemical contaminant by the water column. Moreover, the fish were fed uncontaminated food, but prey items in contaminated areas are likely to be an additional source of chemical contaminants.

The sensitivity of Fulton's K condition index and lipid index was confirmed in our field study with juvenile flounder. Based on Fulton's K condition index, fish from the Canche estuary were found healthier than fish from Scheldt, Seine and Loire estuaries. K index values of fish from the Canche estuary were found to increase with fish size and consequently with the time spent in the area (Fig. 55). This increase of K indices with length was less evident in the three contaminated estuaries than in our reference site with lead to stronger significant differences between reference and contaminated estuaries for the 9–9.9 cm class size than for smaller size. Decrease of fish condition in contaminated estuaries was also showed by the analysis of lipid content in fish muscles. As fractionation of lipid

content into individual classes could provide a more sensitive measurement of metabolically available lipids and energy allocation (Norton et al., 2001), TAG:ST ratios were also measured. Results confirmed the low lipid concentration observed in fish from the three anthropogenic estuaries since TAG:ST ratios were higher in flounder from the Canche estuary.

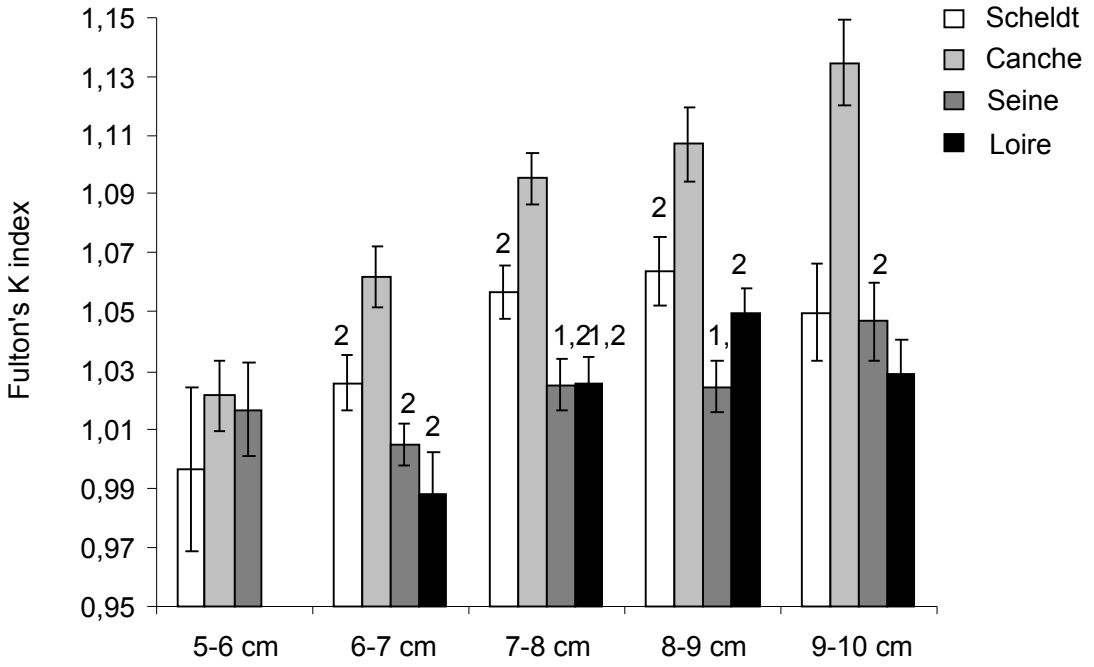


Figure 55: Fulton's K condition index for five size group of flounders sampled in the four estuaries (see Fig. 51). n=30. ([1]), ([2]), ([3]), ([4]) represent significant difference ($p < 0.05$) compared to the Scheldt, the Canche, the Seine and the Loire respectively.

In the wild, fish are subjected to many other stresses (natural or anthropogenic) which could lead to additional effects on those induced by chemical contaminants. This is particularly the case in shallow coastal areas (estuaries, bays, lagoons) that are used by many juvenile fish as nursery grounds. Among the stress faced by these juveniles, the availability and quality of food play a major role in determining growth and overall population size (Gibson, 1994). Decrease in food availability combined with pollutants effects could be critical for juvenile fish since it could increase the amount of energy individual fish need to maintain basic metabolic processes and repair damaged biological systems (Driedger et al., 2009). That's why in a last study, we analyzed the ability of juvenile turbot, *Scophthalmus maximus*, weakened by a chemical contamination, to tolerate restricted feeding conditions.

After 35 days in clean sea water, results show that even turbot growth rates and TAG:ST ratios have highly decreased during exposition to contaminated sediments compared to control, they were picking up until values similar to control fish with optimal feeding condition (Fig. 56). On contrary, with restricted feeding condition, TAG:ST ratios were significantly lower in fish previously exposed to contaminants compared to control fish. Results of metal analysis after the 26 days exposure show a significant increase of hepatic Cd, Cu and Pb concentrations in fish exposed to contaminated sediment compared to control. This metal bioaccumulation was no longer observed after 35 days in clean

seawater with an once a day feeding but still found, for the same elements, with restricted feeding conditions. These results suggest that fish weakened by chemical contaminants would be able to recover good physiological performance when clean environment is getting back but would be affected by limited feeding condition as found during winter.

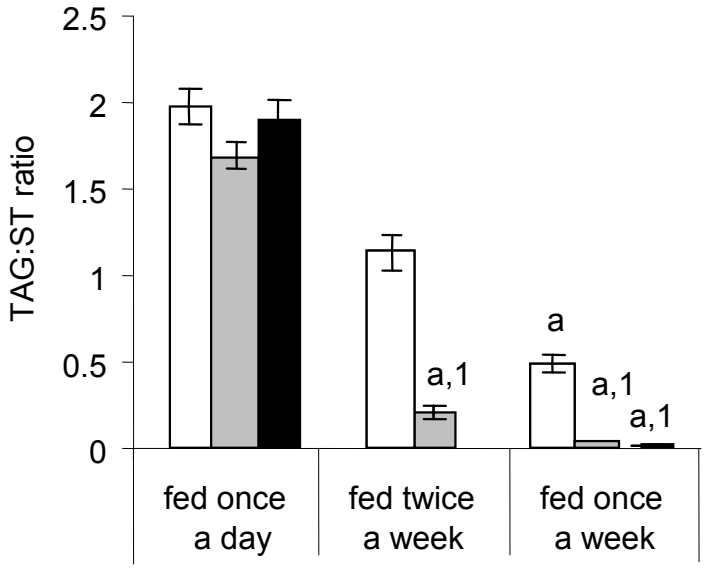


Figure 56. TAG:ST ratios of turbot exposed for 26-days to the three sediments: Ref (□), B (▒) and C (■) (see Fig. 52) after the 35-days recovery period in clean sea water fed once a day, twice a week or once a week. ([1]) represents significant difference ($p<0.05$) compared to reference of the same sample time and ([a]) compared to fish fed once a day among the same treatment.

In all studies, with the exception of the flounder one, the responses of two biotransformation parameters, EROD and GST, and an antioxidant enzyme, the Catalase, were used as early warning tools of toxicity. Relationships between these biochemical biomarkers and physiological ones throughout the different experiments are showed in Table 11.

Table 11 : Synthesis of biomarker responses used in the five studies 1) sea bass exposure to petroleum 2) sea bass and turbot caged in the harbour 3) turbot exposed to contaminated sediments 4) flounder sampled in estuaries and 5) turbot transferred to clean seawater following sediment exposure. Up arrow represent a significant increase for more than one time (↑), two times (↑↑) and three times (↑↑↑) the respective reference value. Similarly, down arrow (↓) represent a significant decrease compared to reference.

Exp.	Species	Contaminants	Method	Site	Time (days)	EROD	GST	CAT	GW	GL	K	ARN:ADN	TAG:ST
1	Seabass	Petroleum	labo		2	↑↑	-	-	-	↓	-	↓	-
					4	↑↑↑	↑↑	-	↓↓	↓	↓	↓	-
2	Seabass	Harbour	caging	Port B	38	↑↑	↑↑	↓↓	↓↓↓	↓↓↓	↓	↓↓	↓↓↓
	Turbot					↑↑	↑	-	↓↓	-	↓	-	↓↓
3	Turbot	Sediment	labo	Port A	7	↑	-	↑↑	↓↓	↓↓	↓	-	
					21	-	-	-	↓	↓↓	-	-	↓↓
				Port B	7	↑↑	-	↑↑↑	↓↓	↓↓	↓	-	
					21	-	↑	↑↑	↓	↓↓↓	↓	-	↓↓
				Port C	7	-	-	↑	↓↓↓	↓↓↓	↓	-	
					21	↑	↓	-	↓↓	↓↓↓	↓	↓	↓↓
				Seine	7	↑	-	↑	↓↓	↓↓	↓	-	
					21	↑	-	-	↓	↓↓	↓	-	-
4	Flounder	Estuaries	*in situ*	Seine							↓		↓↓↓
				Escaut							↓		↓↓↓
				Loire							↓		↓↓↓
5	Turbot	Sediment	labo	Port B	26				↓↓↓	↓↓	↓	-	↓↓
				Port C					↓↓↓	↓↓	↓	-	↓↓
		Fed once a day		Port B	61				-	-	-	-	-
				Port C					-	-	-	-	-
		Fed once a week		Port B					-	-	-	-	↓↓↓
				Port C					-	-	-	-	↓↓↓

In the petroleum study, an increase in EROD activity was observed in the juvenile sea bass after 48 h of exposure (Tab. 12). Sea bass EROD activities doubled after exposure of 96 hours which suggested a higher level of sea bass exposure as compared to the 48 h. GST induction was observed in those sea bass exposed to crude oil, but an increase was only observed after four days of exposure. The 48 h exposure to petroleum appears not to have been sufficient to induce this enzyme. We found no difference in catalase activity between the petroleum exposed sea bass and the control group.

Table 12: Ethoxyresorufin-O-deethylase (EROD, pmol.min^{-1}.mg prot^{-1}), Glutathione S-transferase (GST, µmol.min^{-1}.mg prot^{-1}) and Catalase (CAT, µmol.min^{-1}.mg prot^{-1}) activities measured on control sea bass gill (CTRL) and petroleum-exposed sea bass (OIL) following 48 h and 96 h of exposure. (*) represents significant difference ($p < 0.05$) compared to respective control

	T = 48 h		T = 96 h	
	CTRL	OIL	CTRL	OIL
EROD	0.01 ± 0.01	2.74 ± 0.14*	0.02 ± 0.01	6.84 ± 0.26*
GST	0.29 ± 0.03	0.31 ± 0.02	0.26 ± 0.02	0.67 ± 0.02*
CAT	1.31 ± 0.06	1.45 ± 0.10	1.51 ± 0.10	1.57 ± 0.10

a.

EROD induction was related to the effects on fish health. Indeed, the EROD induction observed after 48 h of exposure was associated with effects on fish growth, and the highest EROD induction after the 96 h period, would correspond to a more important petroleum impact on fish health.

In complex chemical mixtures, such as those found in the polluted sediments of the studied harbour, biomarkers regulations are potentially subject to additive, synergistic or antagonistic chemical interactions. The two biotransformation parameters (EROD and GST) activities were found to be higher in sea bass and turbot, caged in the inner part of the harbour, compared to the station located in front of it. As higher levels of PAHs were detected in station B compared to station A, the increase of these biotransformation enzymes could be explained by these organic compounds. A decrease of catalase activities was observed for both juvenile sea bass and turbot, even though the decrease was not significant for turbot. High levels of both EROD and GST activities were significantly correlated with reductions of the somatic growth rates, RNA:DNA ratios and lipid index of sea bass caged in the contaminated station (Tab. 13). This relation suggests that there are metabolic costs associated with the synthesis of these proteins or with detoxification processes (Rose et al., 2006). The decrease of catalase activities measured in sea bass was also associated with a decrease of all physiological biomarkers. Indeed, catalase activities showed the strongest correlations with growth and condition indices compared to the two biotransformation parameters measured.

Similarly than for the caging experiment, differences in EROD, GST and CAT activities were observed in turbot exposed to the four contaminated sediments compared to the reference. Highest responses were observed in fish exposed to sediment B while sediment C presented the highest concentrations of most of the chemical contaminants. While EROD and CAT showed the highest responses at t_7, a low relationship was found between biochemical biomarker responses at t_7 and the physiological performance of fish. At t_{21}, an EROD increase was found to be significantly and negatively related with RNA:DNA ratios and CAT with both specific growth rates and the Fulton's K index. However, this relationship remained relatively low with a correlation coefficient of about 30%. The main difference between biochemical and physiological biomarkers was in the responses observed in turbot exposed to sediment C. Indeed, few differences in biochemical biomarkers were observed in fish exposed to sediment C while a high difference was observed for condition B (Fig. 57). On the

other hand, the lowest responses of physiological biomarkers were observed for condition C. Sediments B and C presented different levels of contamination among the metals. This fact supports the previously hypothesis of potential antagonist effects of the different chemical contaminants present in sediments on biochemical biomarker activities. The analysis of relationships between biochemical and physiological biomarker responses suggests that, based only on biochemical biomarker responses, biological effects of exposure to sediment C would be underestimated.

Table 13: Pearson's correlations between biomarker responses (EROD, GST, CAT) and biological parameters (specific growth rate in length (GL) and weight (GW), Fulton's condition index (K), RNA:DNA and TAG:ST ratios) measured in juvenile sea bass (a) and turbot (b) caged in stations A and B. Significant correlation for p* < 0.05, p** < 0.01 and p*** < 0.001.

	GW	GL	K	R:D	TAG:ST	EROD	GST
EROD	-0.41*	-0.49*	-0.22	-0.43*	-0.45*	-	
GST	-0.57**	-0.56***	-0.53**	-0.48*	-0.37	0.40*	-
CAT	0.74***	0.70***	0.60***	0.47*	0.63***	-0.30	-0.59***

a.

	GW	GL	K	R/D	TAG:ST	EROD	GST
EROD	-0.45	-0.04	-0.46*	0.04	-0.47*	-	
GST	-0.06	0.15	-0.14	0.19	-0.24	0.27	-
CAT	0.01	-0.20	0.33	0.08	0.62**	-0.50*	-0.25

b.

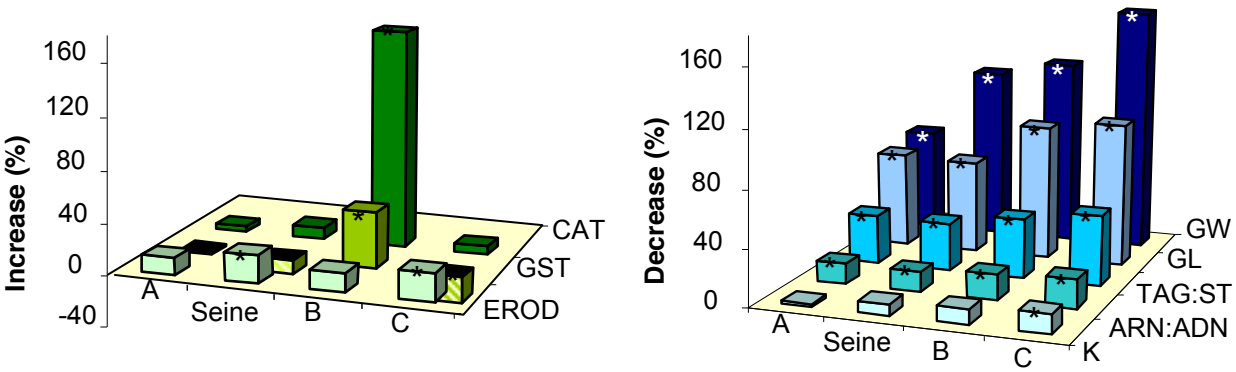


Fig 57 : Variations of biochemical (A) and of physiological (B) biomarkers values (B) in turbot exposed to sediments A, B, C, Seine compared to REF sediment. * <p=0.05.

Ecotoxicological tools applicable to the prediction of chemical contaminant effects have been developed throughout this experimental approach. These data confirm complex cause-effect relationships between exposure to pollutants, metabolisms and heath damages. Whereas responses of biochemical biomarkers appear related to physiological biomarkers ones following an accidental pollution with on type of pollutant, this relationship was less evident with chronic pollution. This inconsistency of biochemical responses measured in this work suggests that variations of biochemical biomarkers can be difficult to predict in a field situation. This study recommends that these biomarkers should not be used alone in biomonitoring programs since interactions between chemical contaminants could alter their responses and lead to an underestimation of biological effects. Indeed, field organisms are exposed to pollutant mixtures including metals and PAHs, which could cause synergistic or antagonist effects on biochemical biomarker responses, whereas physiological biomarkers appear to be more predictive of the adverse effects of chemical contaminants. The use of biomarkers with higher ecological relevance appears essential to evaluate effect of chemical contaminant on organism health and *a posteriori* on populations.

Throughout these field and laboratory studies, growth parameters, Fulton's K index, RNA:DNA ratio and lipid index, based on Triacylglyerol on Sterol ratio, were successfully used to assess effects on chemical contaminants on juvenile fish. They inform indirectly on energetic cost induced by chemical contaminants and directly on growth of the exposed individual (Adams et al., 1990). Thus, they can reflect fish probability to survive. These biological indices present the advantage to be relatively easy to measure and offer relevant information on fish health. That's why present results suggest that these indices could be used in ecotoxicological studies making the assumption that reductions in fish growth and energetic status due to chemical pollution could dramatically decrease their chances for survival in nursery grounds. Nevertheless, the inherent natural variability of physiological biomarkers and their sensitivity to several biotic or abiotic factors (sediment grain size, currents, food availability…) occurring in a field situation has to be taken into account to avoid a misunderstanding of the biological effects of chemical contaminants. That's why, both laboratory and field should be considered to estimate the quality of a given site. In particular, caging experiment could represent a good compromise since using this technique, we were able to integrate true ambient conditions over chemical exposure, which would have been difficult to achieve in laboratory studies. Moreover, this study demonstrates the suitability of using juvenile fish in ecotoxicological studies, particularly since physiological performance have been shown to be higher in younger individuals (Canli and Atli, 2003), probably due in part to the differences in metabolic activity between younger and older fish.

In conclusion, physiological markers used in this study were found to be useful indicators for measuring the condition of the fish. However biochemical markers were only poorly correlated to these physiological indices showing that using only a few biomarkers may not lead to a proper diagnosis. Instead a more developed set of biomarkers should be chosen (see following section) or a carefully selected set of relevant biomarkers should be used in relation to specific aims. It should be noted that chemical measurements such as PAH assessment did not allow either to provide relevant information because they are rapidly metabolized by juvenile fish. This really questions on the choice of the right reference and the pertinent information chemical measurements can provide. Similarly, the physiological indicators used does not allow to draw conclusions on survival or reproduction of the organisms that can adapt to more or less disturbed environments. Thus, the use of a relatively wide biological indices integrating different levels of biological organization range seems the most appropriate approach for a general study of the health of organisms (which does not preclude mechanistic approach for specific problems).

IV.5. Development of a multi-biomarker index

The difficulty in assessing the large amount of data generated by the deployment of a multi-biomarker approach is an argument put forward to justify the uneasy use of these tools in a regulatory context. To fill this gap, several authors have proposed methods for aggregating the results to simplify the reporting of data or to associate the measured response with management guidelines.

As part of this effort, the work was to develop and to test a tool that will synthesise the multi-biomarker results into a simple and easy to understand value. Prior to this work, and based on an analysis of existing tools, several development criteria have been defined:

- the reporting of results must associate a numerical value to quantify the level of impact, and a graphical representation to consider the specificity of the response;
- data processing must be based on the notion of deviation from the reference. This concept, introduced as part of the determination of the ecological status of water bodies in the European Water Framework Directive, will contribute to the suitability of this tool and policies for monitoring environmental quality;
- the developed tool should to be scalable to easily incorporate new indicators and/or allow its transfer to other species.

This work was performed in the three-spined stickleback (Gasterosteus aculeatus L.), a fish model species in ecotoxicology whose potential for application in aquatic biomonitoring is now clearly established (Sanchez et al., 2007, 2008a, 2010).

The developed biomarker index (IBI) is based on the IBR (Integrated Biological Responses) developed by Beliaeff and Burgeot (2002) with a modification of the latter to take account of the criteria set out above. Previously, the data should show a normal distribution or approach. Thus, in a first step, the log data is transformed according to the following relationship:

$$Y_i = \log\left(\frac{X_i}{\overline{X_0}}\right)$$

where Xi is the measured value for a biomarker in an individual sampled at a given site and $\overline{Xo}$ the calculated average for a biomarker of the data used as a reference.

On log-transformed data, it becomes possible to apply the mathematical rules that govern the standard normal distribution and thus to describe the position of each sample relative to the average of the population considered in determining the parameters for each Zi individual according to the following relationship:

$$Z_i = \frac{Y_i - \mu}{\sigma}$$

To create a baseline of 0 and represent the deviation of biomarkers measured on a site in relation to the deviation calculated for the baseline data, a parameter Ai is determined for each biomarker at a given site using the following equation:

$$Ai = \overline{Z_i} - \overline{Z_0}$$

where $\overline{Z_0}$ x is the mean of Zi calculated for the reference values of each biomarker.

Ai values calculated for the different biomarkers at a given site are then plotted on a star chart which then represents changes, both positive and negative, of different biomarkers measured on a site relative to standard reference values (Fig. 58). A numeric value of "biomarkers" (IB) index specific to each site and represents the sum of the effects observed on the site in question, is also calculated using the following equation:

$$IB = \sum |Ai|$$

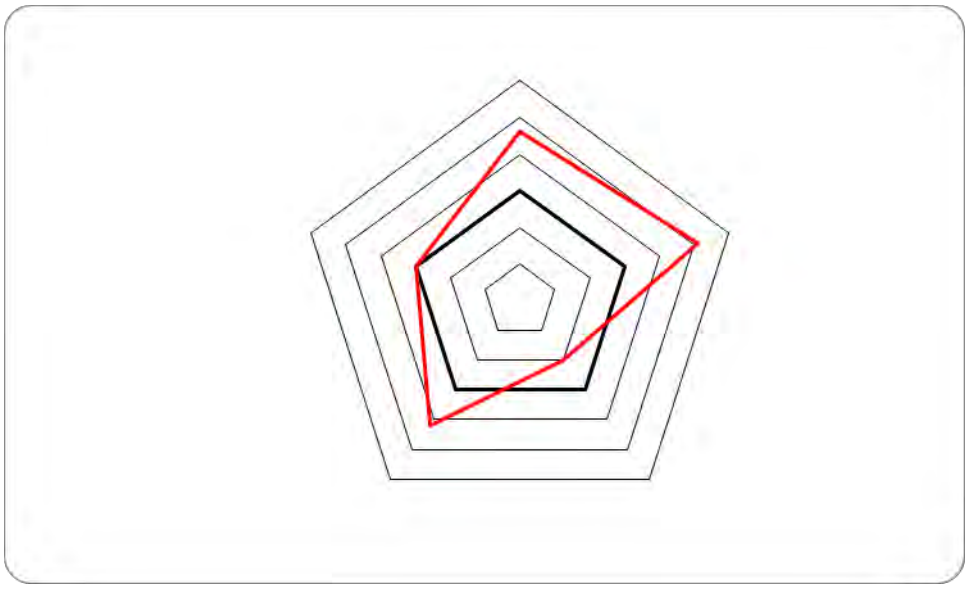


Figure 58: Graphical representation of the biomarker index. The response profile shown is that of a site characterized by induction of EROD, TBARS and VTG but also by inhibition of AChE and no effect for the SPG.

This method raises the question of the selection of the baseline. This is an important parameter in the calculation of the multi-index as biomarkers are directly related to the reporting of results and their interpretation. Thus, several strategies can be viewed with:

- the use of an upstream reference. This strategy can be used for studies of such upstream / downstream or for rivers;
- the use of a historical reference. This strategy, based on available data, is particularly suitable for monitoring a site;
- the use of a so-called "absolute" reference representing the physiological value of the biomarker studied in a given condition. This strategy seems to be the most suitable for the implementation of biomarkers on a large scale.

In this study, the basal values measured in sticklebacks sampled on a low polluted site, Vallon du Vivier (Tancarville, Seine-Maritime, France), were used (Sanchez et al., 2008b). To assess the validity of the reference over a wide geographical area, a sampling campaign was implemented at the national level. It involved eight locations selected for membership in the network control and monitoring of the WFD for their different pressures (Tab. 14). Three selected sites are reference sites of the WFD. This study was completed by the study of two low contaminated sites that are not part of the networks described above: the Vallon du Vivier and Val des Fontaines Tancarville (76).

Table 14: Overview of the study sites and sampling results. * Indicates reference sites.

Site	Date	Effectif
Sorgue à Fontaine de Vaucluse (84)*	28/07/2009	20
Massane à Argelès sur Mer (66)	05/08/2009	13
Ruisseau des Assats à Genihat (63)	20/08/2009	20
Brioude à Gannat (03)	21/08/2009	20
Rhin à Hombourg (68)*	25/08/2009	20
Muehlbach à Landser (68)	25/08/2009	20
Evoissons à Bergicourt (80)*	10/09/2009	18
Lézarde à Epouville (76)	16/09/2009	20

On each of the studied sites, 20 sticklebacks were sampled for the measurement of a set of biomarkers (Tab. 15). In parallel, different biotic factors (length, weight, sex, sexual maturity, parasitism) and abiotic (water temperature, conductivity, oxygen concentration, accompanying species, area Huet rank Strahler) were measured.

Briefly, the results showed that the reproductive status of fish at each sampling period are major confounding factors in the interpretation of results. Therefore it appears important, to limit the extent of a sampling period, or in the case of large-scale studies, to work on individuals with similar stage of reproduction. Under this condition, the variability of the reference line appears to stabilize and allows the interpretation of results. This study also highlight the difficulty posed by the use of reference sites or sites with low anthropic impact to study the natural variations of a biomarker because of the multiple mechanisms of action of pollutants that are measured through a multi-biomarker approach.

Tableau 15 : Biomarkers used to study the variability in baseline values. * Indicates biomarkers used to calculate the IBI.

Biomarqueur	Fonction	Susceptibilité
7-éthoxyrésorufine-O-dééthylase (EROD)*	Biotransformation	HAP, PCB-DL, dioxine
Cytochrome P4503A (CYP3A)	Biotransformation	Pesticides, médicaments
Glutathion-S-Transferase (GST)	Biotransformation	Pesticides, HAP
Glutathion Peroxydase (GPx)	Stress oxydant	Divers
Glutathion total (GSH)	Stress oxydant	Divers
Lipoperoxydation (TBARS)*	Stress oxydant	Divers
Acétylcholinestérase (AChE)*	Transmission nerveuse	Pesticides
Vitellogénine (VTG)*	Reproduction	Xéno-oestrogènes
Spiggin (SPG)*	Reproduction	Xéno-androgènes

An alternative to the definition of the baseline is the use of data acquired on an upstream site. This adjustment is only applicable in the context of an upstream/downstream monitoring for synthesizing multi-biomarker responses at a site of interest (eg downstream of an effluent, ...).

In conclusion, this work has developed a methodology for data standardization of biomarkers and their aggregation into a multi-biomarker index. This tool originally developed in the stickleback can now be applied to other species, and with other biomarkers.

IV.6. Feminisation of wild fish

Under this program, a specific sampling method was developed. This is to provide containers with formalin 4% of personal ONEMA in charge of monitoring fish populations. Thus, immediately after their capture, lots of 30 cyprinids (roach, gudgeon, chub, minnow) are sacrificed formalin then repatriated to INERIS for analysis. On each individual, gonads were dissected and prepared for analysis of intersex as described by Batteman et al (2004). The rat kidney, liver and gills were dissected, embedded in paraffin and stored.

Thus, 16 sites were used for the analysis of intersex. The results show that 9 of them intersex fish have been observed with a case between 3 and 36% (Fig. 59). To complete this analysis, the severity index of intersex previously developed in flounder was measured. This index, which corresponds to a score of 20 varies between 0.3 and 7.1 and showing the different levels of severity that may be encountered in French rivers.

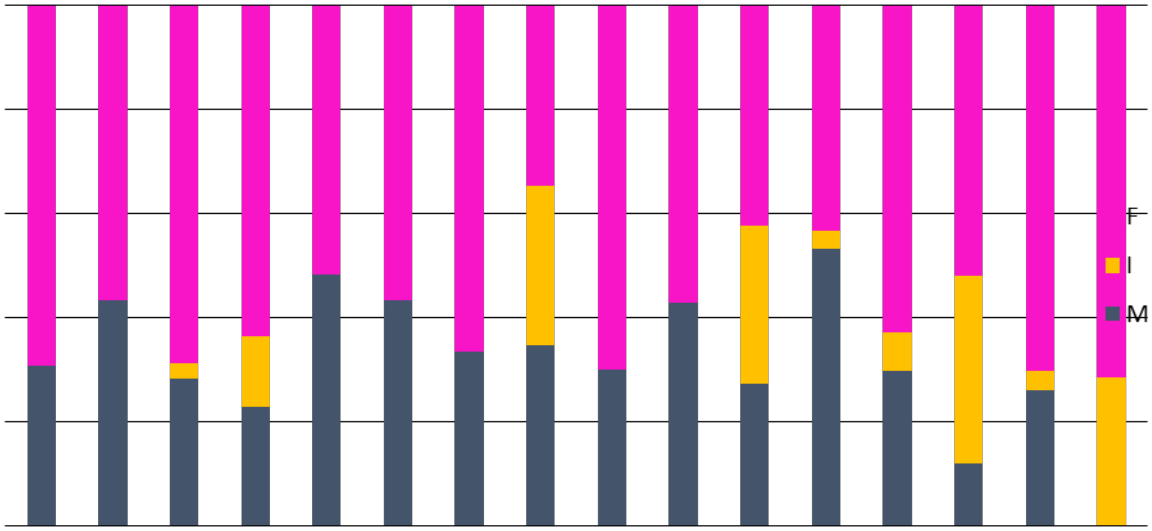


Figure 59 : Occurrence of intersex fish (I) in roach sampled in rivers of the study zone.

During previous INTERREG works, fields sampling were conducted in the Northern part of France. Histological analyses of male gonads allowed to study the occurrence of intersex fish in the rivers (Fig. 60). Several conclusions can be drawn from the results. Highest intersex occurrences were found in the Bray country, a region characterised by its low industrialisation and a moderate human density, which suggest that the agriculture activities may be, at least partly, responsible for the effects on the fish population. The northern part of France display, conversely, a low occurrence of intersex fish despite that sampling were made in industrial areas with high metallic contamination. The Seine and the Somme rivers are hosting a fish population with a moderate percentage of intersex roach (the

highest percentage of intersex fish observed at Triel sur Seine is based on a low number of fish and thus may be considered with caution).

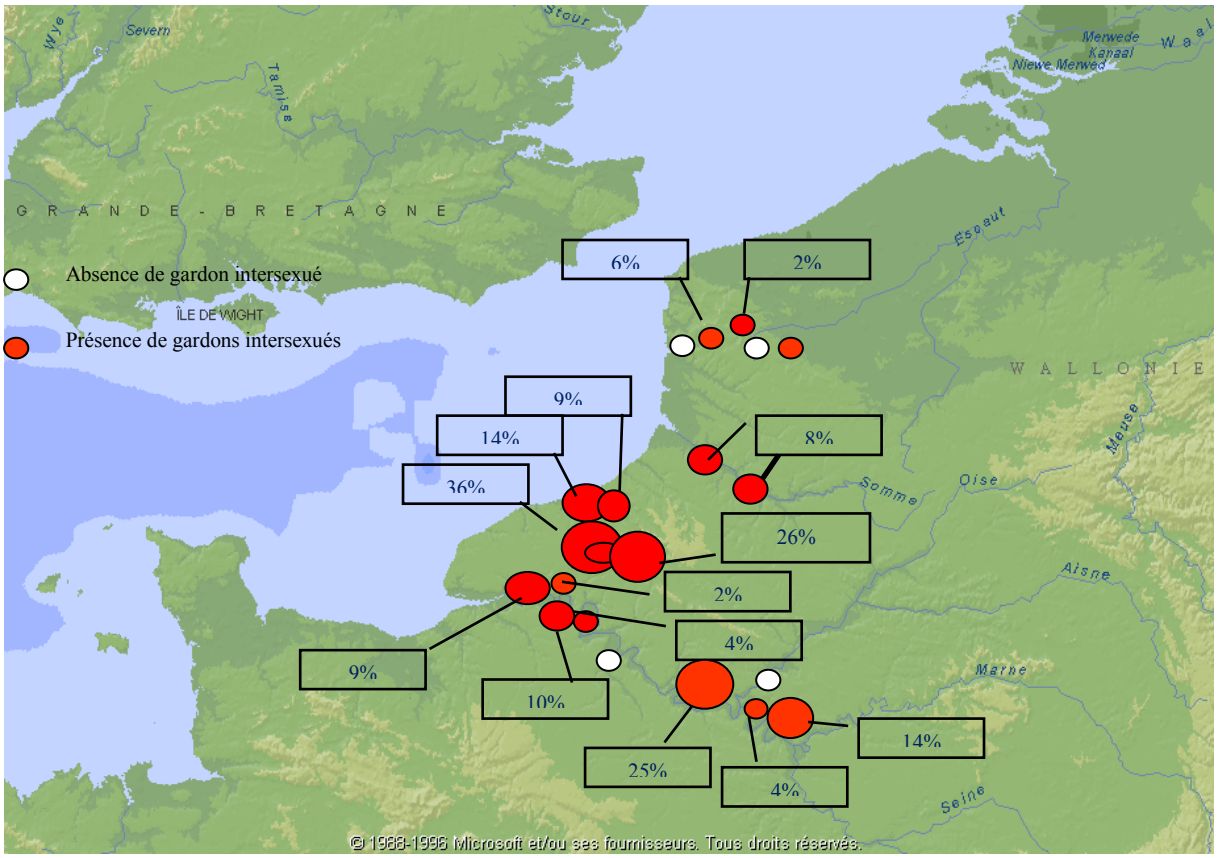


Figure 60 : Map of the occurrence of intersex roach (2000-2010)

In conclusion, the extent of occurrence and severity of intersex roach in French rivers shows that these fish are probably exposed to endocrine-disrupting pollutants in concentrations that varies according to sites. In addition, this study confirms the feasability of measuring a biological indicator reflecting the health of living organisms in relation to their exposure to endocrine-disrupting xenobiotics.

IV.7. Surveys of anti-androgenic activity in coastal sediments

A number of sites were sampled by DIESE partners during 2009-2012 (including main estuaries and coastal regions, Fig. 60). In some estuaries multiple sites were sampled so that the overall survey encompassed a total of >100 sites in France and UK. Sediments were sampled at 2-3 cm depth, and each sample comprised 20 subsamples taken in a 3 m^2 area. At the same time as the sediment collection, up to 30 clams were sampled from each site to determine the incidence of intersexuality and levels of AA activity within the tissues.

A further survey of sediments at 5 sites in the Southampton estuary were taken confirm the high levels of AA activity in this estuary. These samples comprised 3 replicate subsamples of a 3 $metre^2$ area. Sediments were sieved to determine % clay, silt and sand content. Total organic carbon (TOC) was estimated from acid digestion using chromic and sulphuric acid to oxidise organic matter, and samples were titrated against Mohrs salt to estimate organic content.

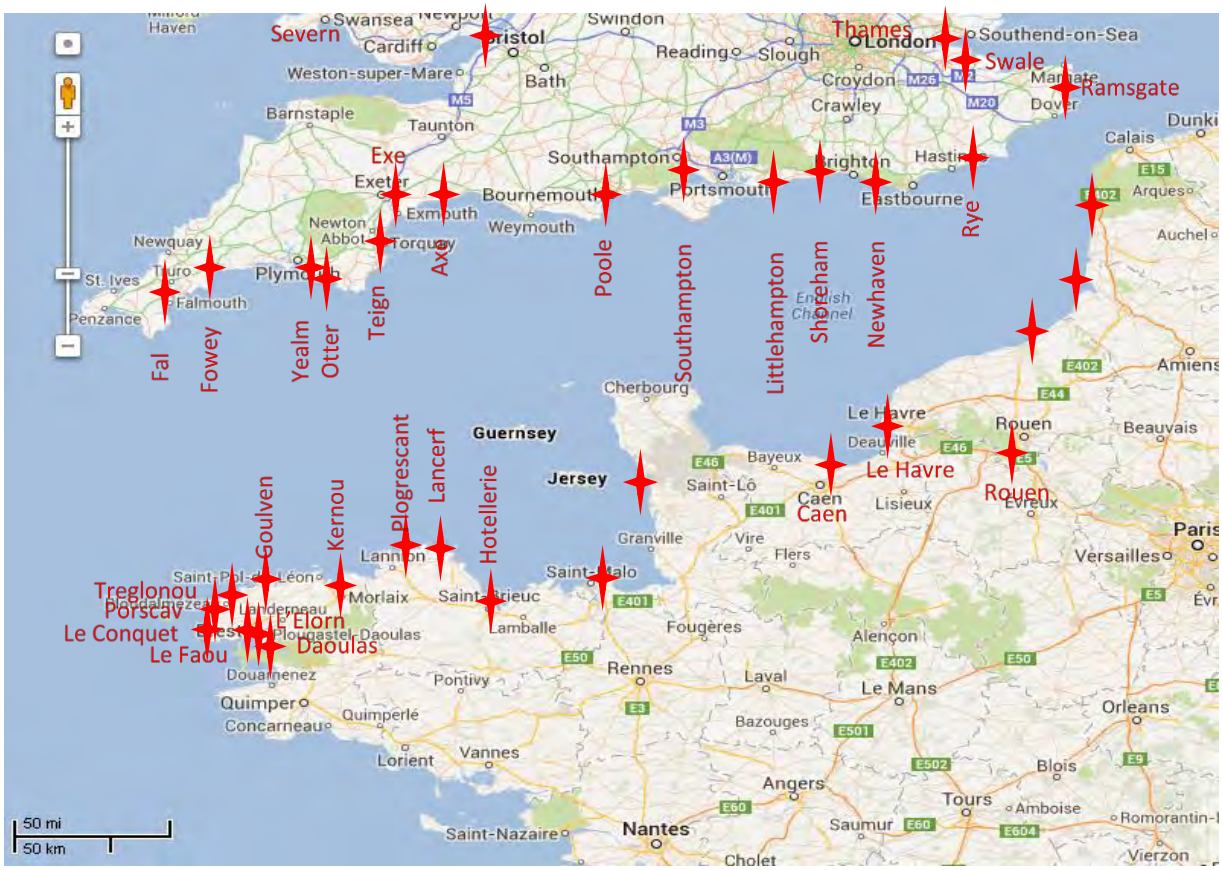


Figure 60. Main sampling sites surveyed for AA activity in 2009-2012

An overview of sediment extraction methodology is given in Figure 61. Briefly sediments were extracted with acetone/methanol using Assisted Solvent Extraction (ASE) methods. The methodology was tested using the AR-CALUX assay to measure AA activity. Results revealed that all the AA activity was extracted using 2 extracts of ASE (see Tab. 16). Each clam was extracted twice with 2 volumes of acetone. The extracts were combined, filtered and resuspended in DMSO for analysis in AR-CALUX.

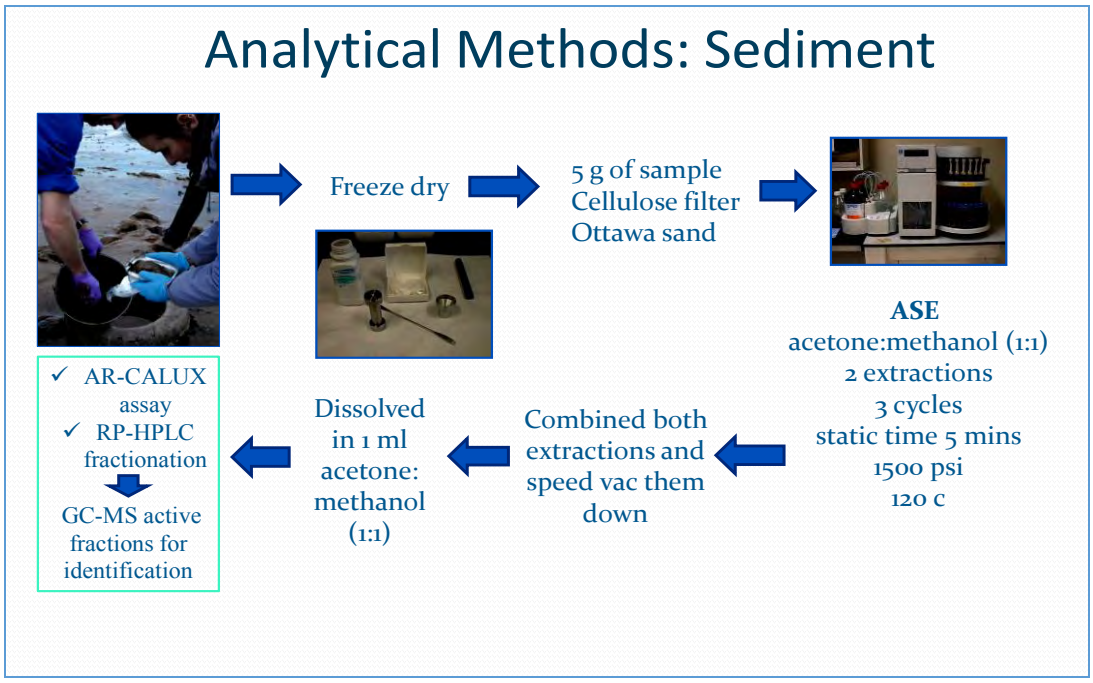


Figure 61. Overview of sediment extraction method and determination of AA activity using AR-CALUX.

Table 16. Extraction of AA activity in sediment samples. Sediments were extracted sequentially with 3 extractions of ASE. All AA activity was recovered in the first 2 extracts. LOD= 0.2 µgFEQ/g dry weight sediment.

Site	Anti-androgen activity ugFEQ/g dry weight sediment	% recovery 1st ASE extraction MEOH/acetone	% recovery 2nd ASE extraction MEOH/acetone	% recovery 3rd ASE extraction DCM/hexane
Sterte	28.6	84.3	15.7	<LOD
Parkstone	48.2	91.9	8.1	<LOD
Minehead	22.5	82.7	17.3	<LOD
Wytch Farm	20.7	74.9	25.1	<LOD
Cracknore	17.0	100	<LOD	<LOD

Levels of AA activity in south UK sediments were between < LOD -220 µg FEQ equivalents/g dry weight of sediment, and in northern France were between <LOD- 73 µg FEQ equivalents/g sediment. The Southampton, Poole, Le Havre estuaries and Rouen were the most contaminated sites (Figure 62 A and B). A repeat sampling in 2013 of the Southampton sites (at Northam and St Denys) confirmed that these sites were the most polluted in the Interreg region.

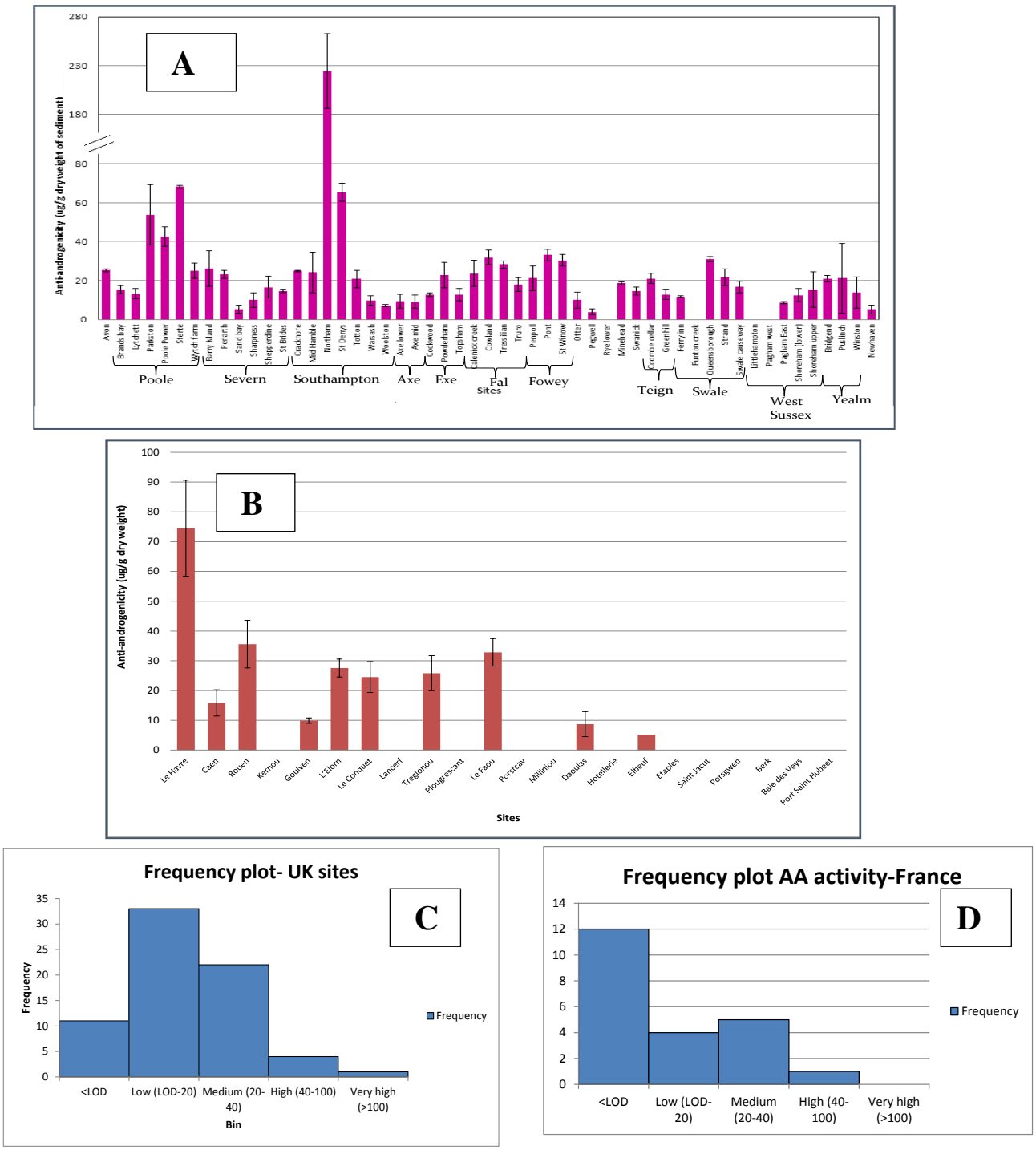


Figure 62. AA activity in coastal sediments of the Tranche Manche region. A) Levels in UK sites. B) Levels in France sites. C) and D) Frequency plots of levels of AA activity in sediments in the Transmanche regions of UK and France.

Frequency diagrams (Figure 62 C and D) revealed that > 88% of sites contained AA activity above the limits of detection (LOD) of 1.0 µg FEQ equivalents/g. In total <5 % of sites would be classified as highly contaminated, with an AA activity of > 40 µg FEQ equivalents/g sediment.

For many contaminants that accumulate in sediments, there is a strong relationship between the levels of accumulation and sediment characteristics such as particle size and TOC. A Kolmogorov-Smirnov test revealed that sediment characteristics and the AA data all followed a non-normal distribution and so Spearman correlation coefficients were used to examine correlations between AA

activity and sediment type. A correlation matrix revealed that AA activity in sediments was strongly positively correlated with % TOC (R^2 0.554) and also, to a lower extent, with % silt content (R^2 0.286) (Table 17, Figure 63). This finding is in keeping with previous reports which indicate that many EDCs, including some anti-androgens present in wastewaters, are moderately hydrophobic and are likely to associate with organic matter present in sediments (Janer et al., 2006). Our results also indicate that % TOC is also strongly associated with the silt fraction which may explain the positive relationship between levels of AA activity and silt content at the different sites (Tab. 17).

Table 17. Spearmans correlation coefficients between AA activity levels in sediments, particle size and % total organic carbon (TOC).

Correlations

		AA activity	TOC	% clay	% sand	% silt
AA activity	Correlation Coefficient	1.000	.554***	.065	-.283**	.286**
	Sig. (2-tailed)	.	.000	.529	.006	.005
	N	95	95	95	95	95
TOC	Correlation Coefficient	.554***	1.000	-.087	-.479***	.583***
	Sig. (2-tailed)	.000	.	.399	.000	.000
	N	95	96	96	96	96
% clay	Correlation Coefficient	.065	-.087	1.000	-.011	-.199
	Sig. (2-tailed)	.529	.399	.	.912	.052
	N	95	96	96	96	96
% sand	Correlation Coefficient	-.283**	-.479***	-.011	1.000	-.748**
	Sig. (2-tailed)	.006	.000	.912	.	.000
	N	95	96	96	96	96
% silt	Correlation Coefficient	.286**	.583***	-.199	-.748***	1.000
	Sig. (2-tailed)	.005	.000	.052	.000	.
	N	95	96	96	96	96

. Correlation is significant at the 0.01 level, * at < 0.001 level (2-tailed).

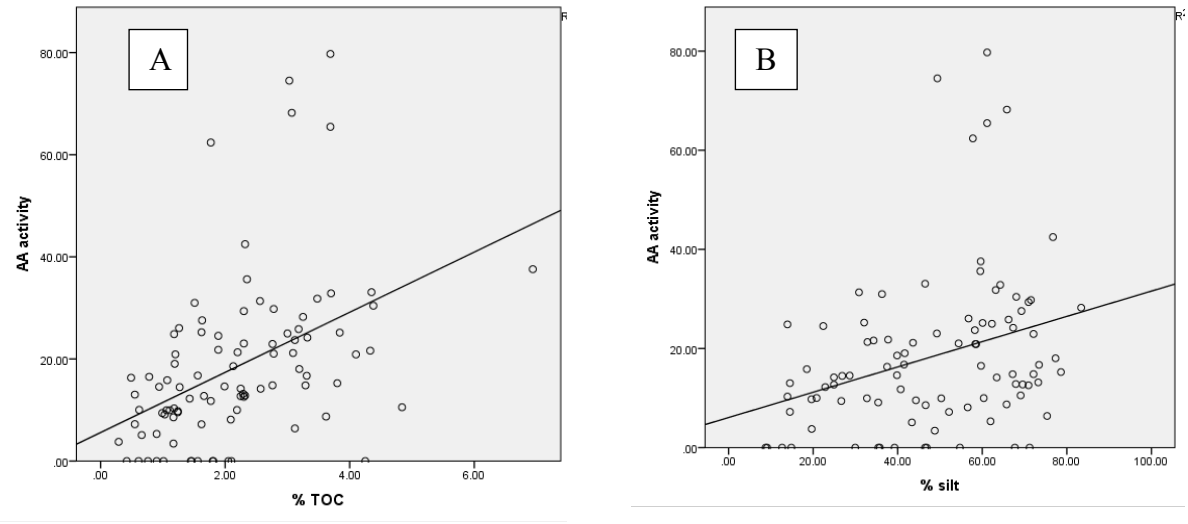


Figure 63. The relationship between levels of AA activity (µg FEQ/g sediment) and A) % TOC and B) % silt in sediment samples. The two highest data points for AA activity (> 150 µg FEQ/g sediment were omitted from the data).

Further ongoing work has revealed that estrogenic activity was detected at most UK sites at levels between 120-353 pg estradiol equivalents/ g dry weight of sediment. On the UK side, both Poole and Southampton estuaries were significantly contaminated with both estrogenic and antiandrogenic activities. Our results indicate that there is significant pollution with EDCs at many coastal sites. These contaminants have the potential to demasculinize marine biota, and may pose a threat to the normal reproductive development of marine benthos.

IV.8. Invertebrate screening tools for endocrine disruption in Channel estuaries and coasts

IV.8.1. Background

Imposex is the imposition of male characteristics (e.g. penis and vas deferens development) on female gonochoristic gastropods and in dogwhelks *Nucella lapillus* is initiated by TBT concentrations as low as 0.5ng l-1. This prevents egg-laying and, prior to TBT legislation, lead to localised extinction of populations.

Figure 64: *Nucella lapillus* (left) – used to measure imposex and *Scrobicularia plana* (right) - an indicator of intersex

Intersex (ovotestis) is the simultaneous presence of both testicular and ovarian tissue in the gonad - to varying degrees. Most intersex invertebrates such as the clam *Scrobicularia plana* (studied in DIESE), and fish, tend to contain small numbers of female germ cells, or oocytes, within a predominantly male gonad (testis), though in severe cases large areas of ovarian tissue may be separated clearly from testicular tissue. In some species there may also be disruption, malformation and blockage of the male reproductive ducts and a female like duct or ovarian cavity

TBT induced imposex in marine snails remains the most remarkable example of the threat of endocrine disruption on Channel ecosystems. Negative aspects of TBT from antifouling were suspected in the late 1960s when it was realised that the release of organotin into aquatic environments was impacting non-target organisms. In the late 1970s and early 1980s, oyster (*Crassostrea gigas*) crops in Arcachon Bay, France, failed, leading to legislation against TBT. Coinciding with this period, work at the MBA showed that TBT was an exceptional endocrine disruptor in marine stenoglossan gastropod species (notably *Nucella lapillus*) causing masculinisation (imposex) in females and widespread population decline. The sensitivity of this and other marine gastropods world-wide has led to their use as bioindicators of TBT pollution. The widespread recognition of effects promoted the spread of legislation and by the early 1990s, many nations had partial TBT bans in place. However TBT is deposited in benthic sediments, where it can last unaltered for decades. Coastal TBT pollution has declined along many shores where legislation was put in place in the 1980s-1990s but remains a concern in various locations because of its persistence in sediments, resuspension from dredged material and occasional illegal usage. Recognition by the International Maritime Organisation that marine pollution does not recognise national, regional or conservation boundaries set the stage for the development of the antifouling convention (2001) with the last date for the application of organotin paints set as 2003 and a total phase out by 2008. The effect of these legislative measures on reducing the threat of ED should by now be anticipated to have taken effect in areas such as the channel and to

test this hypothesis we have reconstructed trends in imposex in *Nucella lapillus* at selected sites over the last quarter of a century.

The broader risk and relevance of endocrine disruption in the marine/estuarine environment, from oestrogenic (feminising) chemicals is not clear and has often been overlooked in invertebrates. High on the list of priorities under biologically-oriented directives such as WFD is the requirement for more information on the symptoms and likelihood of impact in field populations, including those associated with sediment, where oestrogenicity may predominate (Langston et al 2007). The focus of our studies has been the important estuarine clam *Scrobicularia plana*. We now have evidence from the DIESE project that disruption to the 'normal' gonadal development of male *S. plana* may be occurring extensively along UK and French coasts, with populations exhibiting varying degrees of ovotestis (an intersex condition typified by the simultaneous presence of both testicular and ovarian tissue in the male gonad, and analogous to the condition seen widely in UK rivers in fish such as roach). We have thus developed a valuable and practical model for screening Endocrine Disruption, in field populations of *S.plana*, and to interrogate, further, both spatial and temporal trends - and the links with anthropogenic causes.

IV.8.2. Imposex and TBT analysis in the Dog-whelk, Nucella lapillus, at selected UK sites

Now, four years after the ban on TBT, and 25 years after initial restrictions on leisure craft, we wish to establish if previously affected sites within the Channel region have been re-colonised by dogwhelks *Nucella lapillus*, and, if imposex is still evident, where is it highest? We also wish to establish the baseline status of these stenoglossan gastropods at less-affected reference sites, using this most sensitive and specific of bioindicators of endocrine disruption (androgenic).

In addition to remarkable properties as an indicator of intersex, *N. lapillus* has other attributes as an environmental indicator in terms of widespread distribution (on exposed and sheltered rocky shores on both sides of the North Atlantic, from Iceland in the North to Portugal in the South). It is found throughout the Channel wherever there is suitable habitat. In addition to extreme sensitivity to TBT, populations also appear vulnerable to eutrophication and algal blooms

During the DIESE project, populations of *N. lapillus* were studied at selected sites in the western Channel region (Figure 65) –at Plymouth (4 sites), Falmouth (3 sites), North Cornwall Coast – Bude and Widemouth (2 sites), plus Jersey (a reference site in mid-Channel) - further sites were added in 2013 (Torbay x2, and two more from the North Cornwall coast). For most of these sites, data collected in 2012/2013 are compared with of previous surveys, notably those described by Gibbs et al., 1987.

The coast of southwest England has many harbours and marinas which host considerable numbers of leisure vessels and many sites here were impacted by TBT-induced imposex prior to 1987 legislation (Bryan et al 1986). The major ports of Falmouth and Plymouth, displaying some of the most severe population effects, are also situated in the region providing suitable 'worst-case scenarios' for long-term study (despite being sited within designated European Marine Sites). These long term surveillance positions include Castle Drive, seaward of Falmouth Dockyard, and Renney Rocks, adjacent to Plymouth Sound (influenced by various boat facilities located within the estuaries of the Tamar and Plym). Studies here were started in 1985 as part of a broad survey to gain evidence of the widespread nature of imposex and the trends in its development. As a contrast, reference sites at Bude and Widemouth on the north Cornwall coast - which is much less impacted by boating or shipping activity and far removed from any major source of TBT pollution - have been scrutinised at intervals over the last 25 years, including in 2012 as part of the DIESE project.

The link between TBT and imposex in *N. lapillus* was established in experiments at the MBA's Plymouth Laboratory some 25 years ago (Bryan et al 1986; Gibbs et al, 1987). The imposex indices introduced (the Relative penis size index (RPSI) and the Vas deferens sequence index (VDSI)) are now used worldwide and are recommended by the WFD, Fisheries Research Services (FRS) and OSPAR (2008) as a means of monitoring water quality. During the course of its ecotoxicology program, MBA has collected long-term data of imposex and TBT burdens in populations of *N. lapillus*

at a number of sites in the English Channel region (UK). As part of the DIESE project some of these populations were re-surveyed during 2012 and dogwhelks analysed for both imposex and tissue TBT levels, using the same methods to chart the progress and timescales of recovery from this most significant example of endocrine disruption in the marine environment.

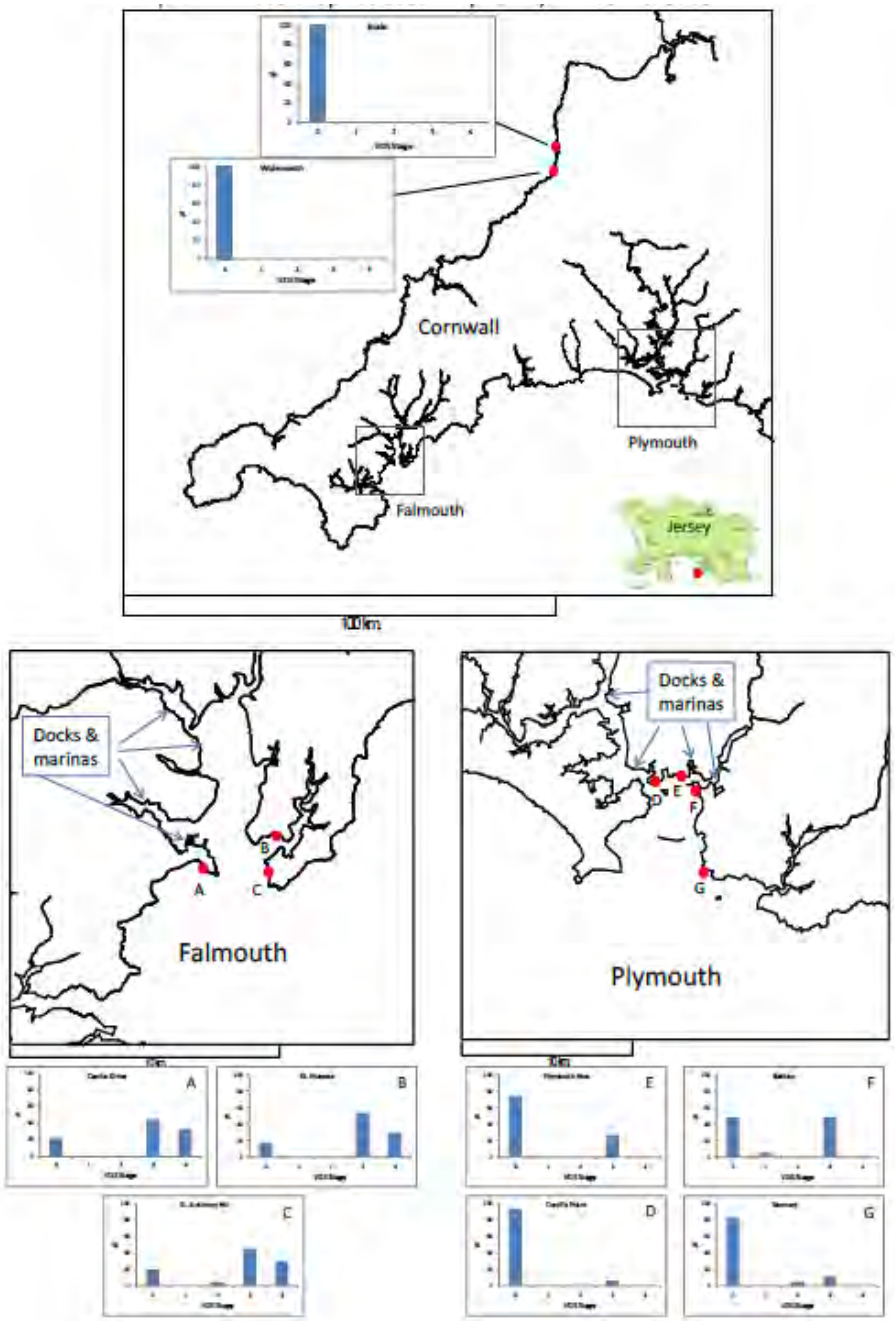


Figure 65: Maps showing location of *Nucella lapillus* sampling sites in SW England and VDS stages

Imposex in dogwhelks may develop at an early age and juvenile females appear to be the most sensitive indicators of short-term contamination. However as *N. lapillus* is a long-lived species (6+ years) and imposex is irreversible, the condition of adults may be more representative of response to long-term exposure to TBT contamination. Hence, 30 to 40 adult dog-whelks were collected from most sites, the shells removed by cracking in a vice, and each was examined with a binocular microscope. The animals can normally be sexed by noting the presence or absence of a penis, or a noticeable penis size differential, and the presence of the reddish-brown sperm ingesting gland in the females. The roof of the mantle is cut open to expose the mantle cavity and allow examination of the

area between the head and the capsule gland or prostate. The length of the penis (if present) is measured in both sexes to the nearest 0.1 mm, from the tip to its base. In females, the area between the penis (if present) and the genital papilla at the anterior end of the capsule gland is carefully examined by rotating the gland to expose its medial surface. The extent of any vas deferens growth is noted, and a numerical VDS stage is assigned (1-6). Several females in some populations had a small penis (usually < 1 mm) but no sign of a vas deferens, either proximally or distally. As the penis was distinct and measurable, these were recorded as VDS stage 3.

The two indices recorded are the Relative penis size index (RPSI) and the Vas deferens sequence index (VDSI). The RPSI is calculated by (mean female penis length3 / mean male penis length3) x 100, and the VDSI as the mean VDS stage of the females (i.e. total VDS scores in females / number of females examined). An additional measure based on the VDSI and given in the Water Framework Directive, is the Ecological Quality Ratio (EQR) which is calculated thus: EQR = (6 - VDSI) ÷ 6, which gives a range of values from 0 to 1.

Size –frequency distribution was measured in the *Nucella* population (n~100) in front of the MBA laboratory on Plymouth Hoe - to compare with distributions in 1967 (pre-TBT) and in 1985 (from Drakes Island, 1km distant from the Hoe since the population here had been eliminated at the height of the TBT threat) (Bryan et al 1986). Generally, populations without juveniles indicate an aging population with little recruitment (i.e. affected by imposex) whilst, in contrast a broad size frequency spectrum is indicative of a breeding, sustainable population.

Subsamples of dogwhelks used in imposex scoring were frozen for organotin analysis (n=6, usually; males and females occasionally analysed separately). Methods for organotin analysis in tissues are modified from the scheme described in Langston et al., 1994. Briefly, for the determination of tin as tributyltin or dibutyltin the tissues of 4-6 frozen specimens were pooled and homogenised. Three aliquots, were placed in glass tubes. A standard of tin as tributyltin oxide in ethanol was added to one sample and a standard of dibutyltin dichloride to another. Hydrochloric acid was added to the tubes, which were shaken for 30min. Organotins were extracted in hexane (15min) and centrifuged. The clear hexane extract was shaken with 1M sodium hydroxide solution to separate the dibutyltin from the tributyltin fraction. Organotins, as tin, were measured by atomic absorption. Detection limits for TBT and DBT in tissues were of the order of 10 ng Sn g-1 dry weight. To convert from Sn to the TBT (or DBT) ion multiply by 2.44 (or 1.96). The method for tissues was also employed on surface sediments in subsequent sections.

The accuracy of analytical methods has been established using either sediment or tissue reference materials, for example, PACS-1. Recoveries of TBT and DBT in spiked *N. lapillus* were 101% and 92% respectively and were corrected by the use of standard additions on all samples.

During the TBT zenith, RPS values in SW England remote from centres of boating activity were 5% or less, increasing to 40% near harbours. VDS stages 1-3 were characteristic of clean areas, whilst stages 4-6 typified sites close to harbours and marinas (Gibbs et al 1987). However even at 'clean' north coast sites (devoid of stages 5 or above) populations exhibited moderate imposex (53-88% at stage 3&4) during the 1980s and surprisingly few females (<1%) were without imposex. These populations were however able to breed, unlike the sterile populations of Plymouth Sound (Batten Bay). In extreme cases this led to extinction (Plymouth Hoe site). Any population with a VDS index above 4 is likely to contain sterile females and may thus have a reduced reproductive capacity.

Current (2012) levels of imposex are summarised in table 18 which shows RPSI, VDS, and % females with penis. Table 19 illustrates TBT and DBT concentrations in tissues of female and male *N. lapillus*.

The proportion of females displaying imposex ranged from 0% (Widemouth) to >75% at Falmouth. However, it is noteworthy that although several females from the three Falmouth sites (Castle Drive, St Anthony Head, St Mawes) were assigned a VDS stage 4, no females in any population were confirmed as sterile. There is now no trace of imposex, at Bude or Widemouth, and only very low levels in Jersey, mid Channel.

Table 18: *Nucella lapillus* imposex values in 2012

Site	male penis			female penis			VDS stages					VDSI	RPSI	EQR	percent with penis	percent female
	n	length	sd	n	length	sd	0	1	2	3	4					
Renney	17	3.30	0.38	18	0.08	0.19	15		1	2		0.0005	0.44	0.93	11.1	51.4
Tinside	11	3.57	0.42	19	0.16	0.33	14			5		0.0126	0.79	0.87	26.3	63.3
Devils Pt	15	2.82	0.43	15	0.03	0.13	14			1		0.0002	0.2	0.97	6.7	50.0
Batten	9	3.32	0.41	21	0.18	0.22	11	1		9		0.01	1.48	0.75	42.8	70.0
Castle Drive	26	2.83	0.32	9	0.77	0.53	2			4	3	2.46	2.67	0.56	77.8	25.7
St Anthony Hd	10	3.34	0.13	20	0.7	0.51	3		2	9	6	0.98	2.65	0.56	75.0	66.7
St Mawes	13	3.07	0.9	17	1.07	0.64	3			9	5	4.24	2.76	0.54	82.3	56.7
Bude	18	3.36	0.44	12	0	0	12					0	0	1	0.0	40.0
Widemouth	8	3.53	0.63	22	0	0	22					0	0	1	0.0	73.3
Meadfoot	13	3.76	0.26	22	0	0	22					0	0	1	0.0	62.9
Saltern Cove	16	3.82	0.32	19	0.21	0.35	12		1	5	1	0.015	1.10	0.62	31.6	54.3
St Agnes	12	4.09	0.38	23	0.06	0.29	21		1		1	0.0003	0.26	0.96	4.3	65.7
Porth Joke	14	4.03	0.21	21	0	0	21					0	0	1	0.0	60.0
Jersey	50	3.13	0.27	40	0.18	0.37	30		1	8	1	0.02	0.75	0.875	22.5	44.4

Table 19: *Nucella lapillus* TBT and DBT values in 2012

	Tissue organotin (µg Sn/g dry wt)							
Site	Female				Male			
	TBT	DBT	Total	%TBT	TBT	DBT	Total	%TBT
Renney								
Tinside	0.0332	0.0098	0.0429	77.3	0.0098	0.0103	0.0201	48.8
Devils Pt	0.0067	0.0090	0.0157	42.9	0.0015	0.0098	0.0098	15.2
Batten	0.0124	0.0148	0.0272	45.5	0.0282	0.0211	0.0493	57.2
Castle Drive	0.0257	0.0250	0.0517	51.6	0.0308	0.0189	0.0497	61.9
St Anthony Hd	0.0285	0.0229	0.0514	55.4	0.0118	0.0197	0.0315	37.5
St Mawes	0.0367	0.0207	0.0574	64.0	0.1503	0.0550	0.2054	73.2
Bude	0.0050	0.0040	0.0091	55.5	0.0207	0.0059	0.0266	77.9
Widemouth	0.0498	0.0076	0.0574	86.7	0.0317	0.0040	0.0358	88.8
Jersey	0.0064	0.0038	0.0102	62.8	0.0252	0.0012	0.0263	95.6

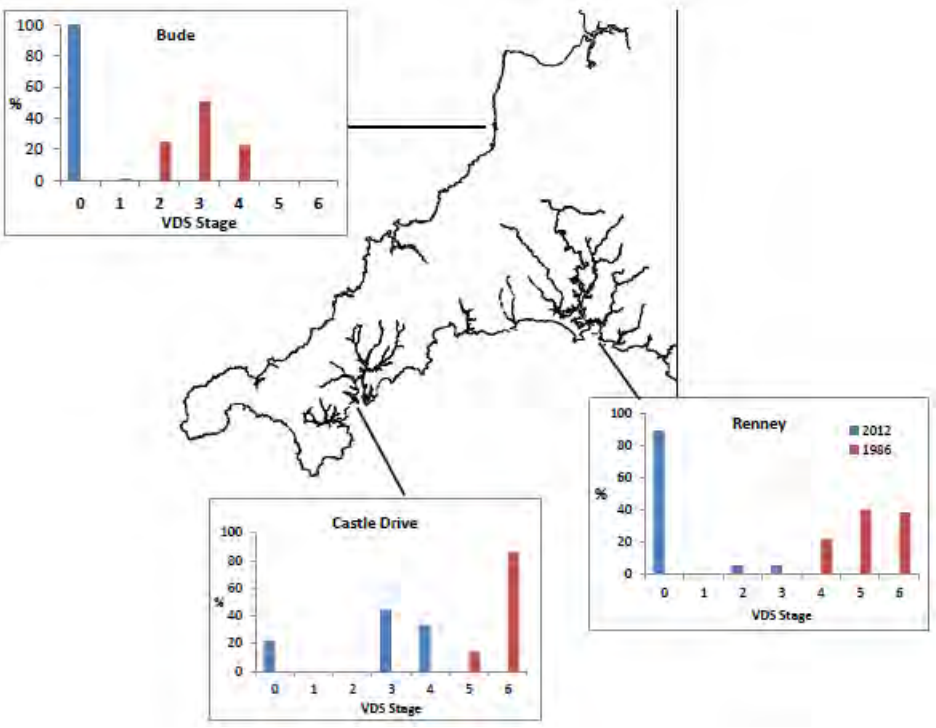


Figure 66: *Nucella lapillus*. Comparison of how the distribution of VDS stages within three SW populations have changed from 1986 to 2012

Detailed temporal changes in VDSI for Renney Rocks (outside Plymouth Sound) are shown in Fig. 67. The majority of change has occurred over the last six years

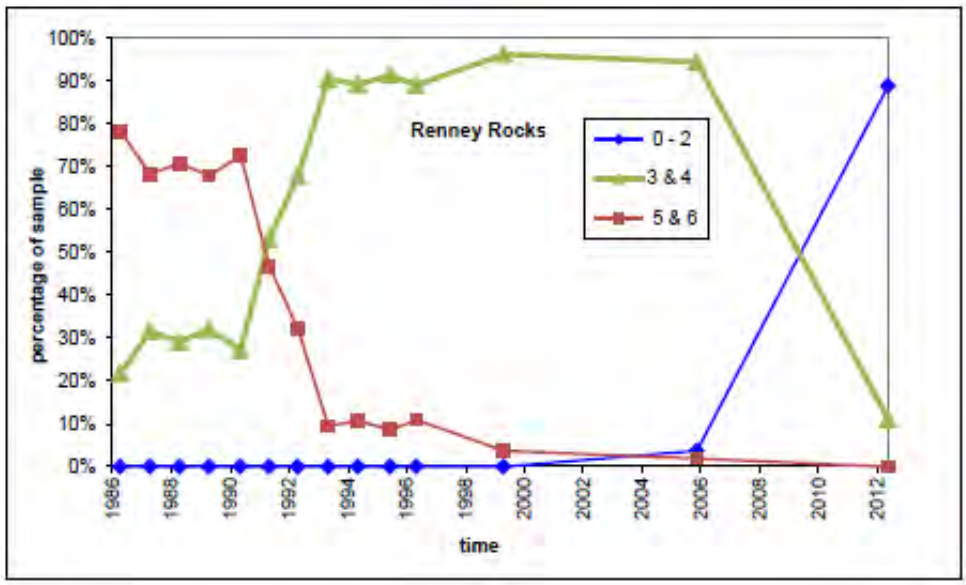


Figure 67. *Nucella lapillus*. Changes in proportion of different VDS stages in the Renney Rocks population during the last 25 years

Comparison of time series for Castle Drive, Renney, Bude and Widemouth confirm significant improvements in imposex (mean VDSI, Fig. 68) and TBT body burdens (Castle Drive, Fig. 69). Nevertheless improvement is clearly much slower at Falmouth (Castle Drive) where the presence of dockyards and sediment reservoirs of TBT may be delaying recovery.

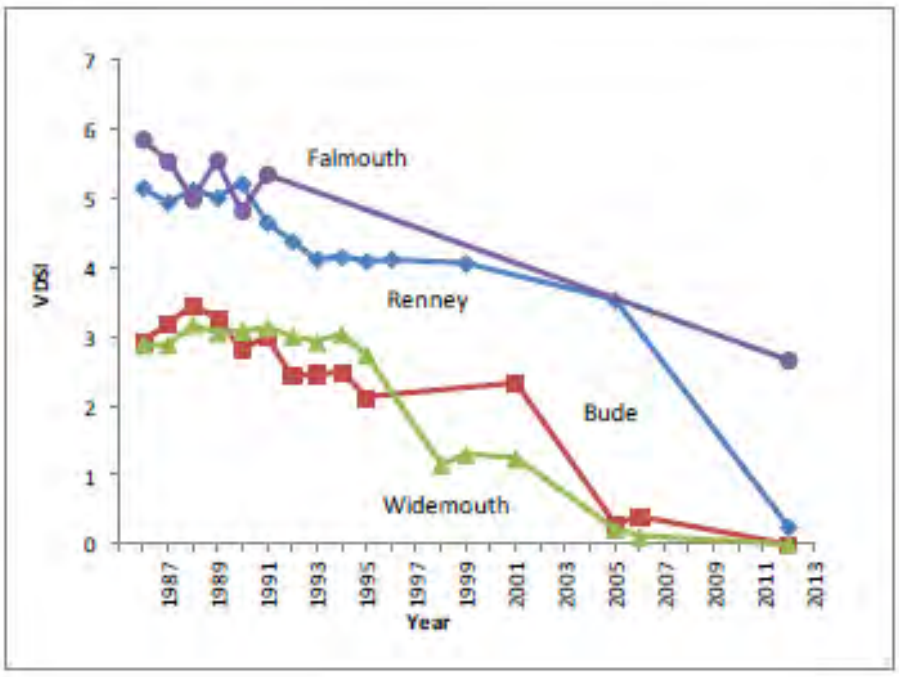


Figure 68: *Nucella lapillus*. Changes in mean VDSI at Falmouth, Renney Rocks, Bude and Widemouth during the last 25 years.

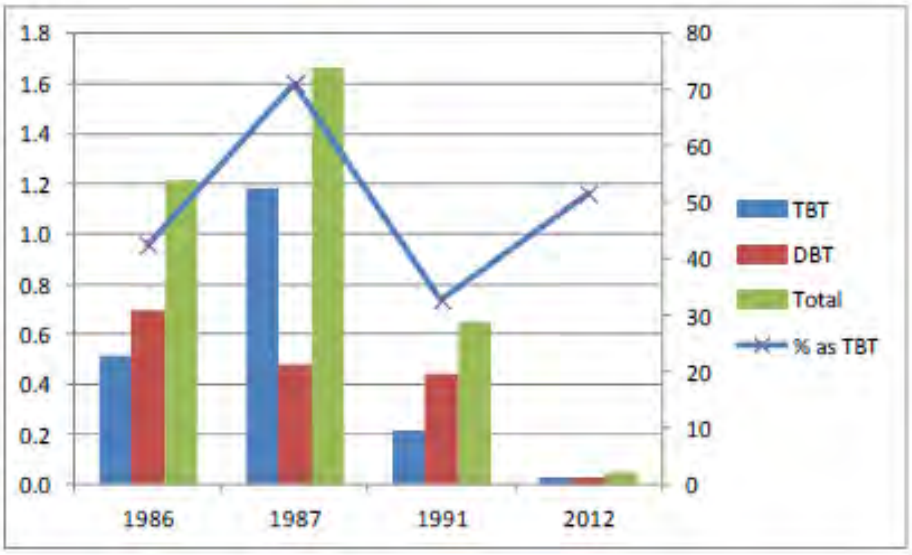


Figure 69: *Nucella lapillus* change in tissue organotin burdens in adults from Castle Drive, Falmouth (µg Sn/g dry wt, left Y-axis; % organotin as TBT, right Y-axis).

The relationship between tissue TBT levels and the VDSI expressed by adult females has been shown to correlate closely (Gibbs et al., 1987). Although the correlation is less significant in recent samples (as tissue concentrations are now comparatively low), the relationship is still evident (Fig. 70).

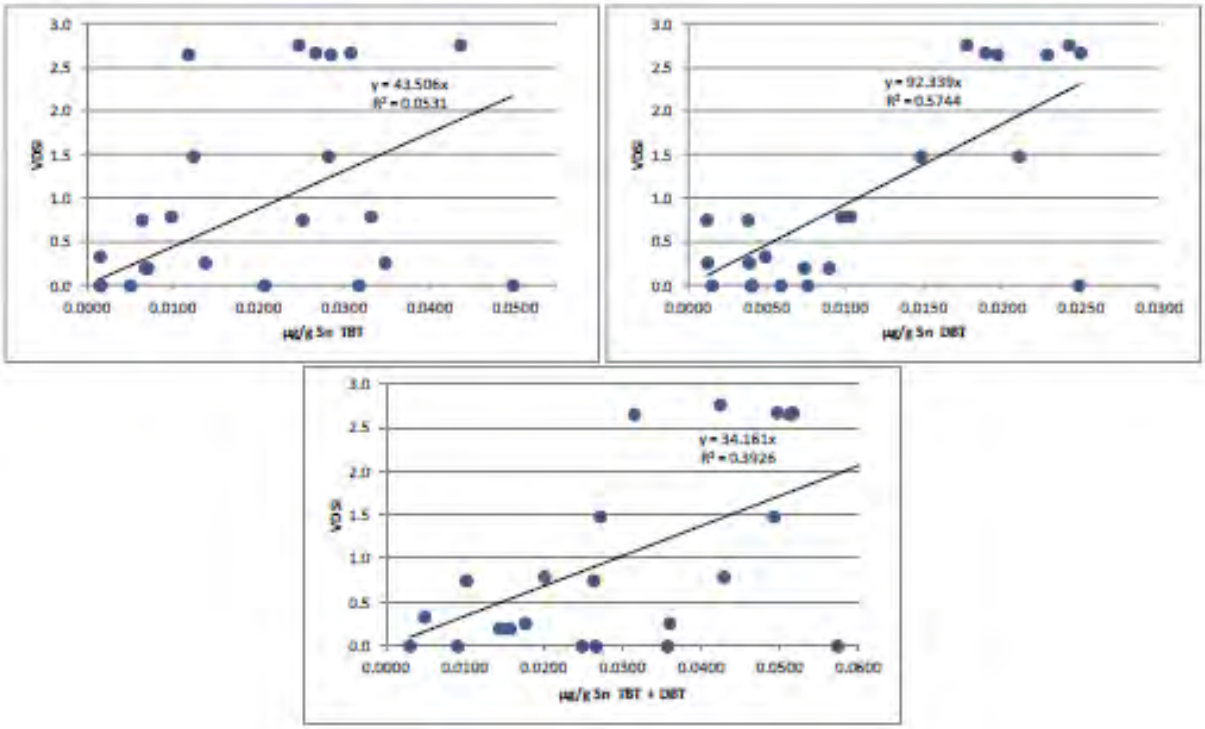


Figure 70: *Nucella lapillus* Relationship between VDSI and tissue organotin burdens (TBT, DBT separately and in combination) in populations from SW England, 2012

Size –frequency distributions in the *Nucella* population at Plymouth Hoe in 1967 (pre-TBT), in 1985 (from Drakes Island, 1km distant, since the population had been eliminated at the Hoe site at the height of the TBT threat) (Bryan et al 1986), and in 2013 are shown in figure 71. Generally, populations without juveniles indicate an aging population with little recruitment (i.e. affected by imposex) whilst, in contrast a broad size frequency spectrum is indicative of a breeding, sustainable population.

Thus, the Plymouth dogwhelk population has now almost recovered from its mid- 1980s slump to its earlier pre-TBT status, with clear signs of recruitment of juveniles. Again this implies that the timescale for impact through to recovery has been of the order of a quarter of a century.

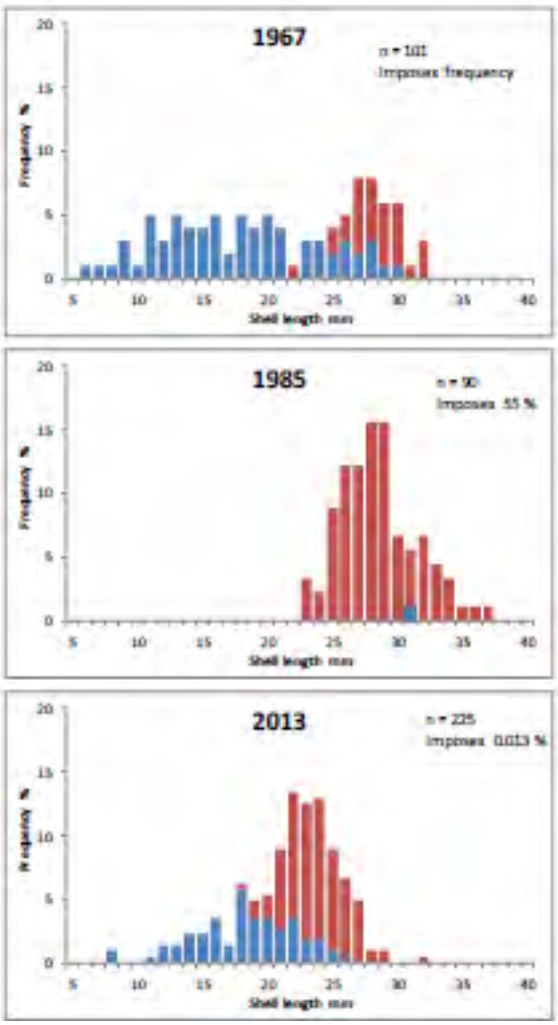


Figure 71: *Nucella lapillus*. Size –frequency distributions in the population at Plymouth Hoe over a 25year period- encompassing the 'TBT years'.

Recovery from imposex and recolonisation of populations following legislation may be anticipated to be slow due respectively to the irreversible nature of the condition in individuals and also as a result of the reproductive cycle of *N.lapillus* (lack of planktonic phase and hence poor dispersal capability). Because of difficulty in collection of population data the most widely measured aspect of recovery is imposex. Results presented here shows clear evidence of the effectiveness of TBT legislation and most sites would achieve the OSPAR Ecological Quality Objective for imposex in *N.lapillus* of a VDSI< 2. Only two sites failed this conservation objective- Castle Drive and St Mawes in the Fal Estuary; (note there other sites in this system where dogwhelks have yet to become re-established following elimination during the 1970s and 1980s).

Assembled data on trends in TBT-specific effects throughout the OSPAR region, published in 2008 (OSPAR, 2008), suggest that intensity of intersex was predominantly of a scale shown in our study (majority of samples with VDS in the range 0.3-5) and that this is decreasing at many sites: 24 out of 134 gastropod time series exhibited a significant downward trend, presumably as a result of legislation banning the use of TBT in the 1980s. Several authors (Colson and Hughes, 2004; Huet et al., 2004; Birchenough et al., 2002; Moore et al., 2000; Bray and Herbert, 1998; Evans et al., 1994, 1995, 1996) included time series in Brittany - relevant to the current Channel project. Thus, imposex levels in the Bay of Brest suggested significant, but declining levels of impact, between 1992-2002, coinciding broadly with lower TBT concentrations in seawater (Huet et al., 2004) though here, as at the majority of sites, comparable time-series on TBT body burdens in *N.lapillus* are much less numerous, making our data distinctive. The overall relationship between TBT body burden and imposex in *N.lapillus*, for sites in SW England (summarised in figure 70) show correlations consistent with initial relationships described in Gibbs et al 1987.

In the context of linking TBT distributions to consequences, attention is also drawn to the TBT time-series for estuarine water, sediment and biota described for Southampton and Poole described in an earlier DIESE report (see also below). These studies confirmed that contaminated sediments in particular could remain a problem for many years (perhaps 20-30) despite the global ban (*see* Langston et al 1994). Dumping sites for such TBT-enriched material have also been shown to impact on intersex levels in gastropods at offshore sites (Santos et al., 2004). This has been one of the considerations which has prompted the adoption of National Action Levels for TBT in Dredged Materials.

It has been suggested that TBT legislation has led to a more rapid recovery in the UK than in other countries such as France, Spain and Portugal (Ruiz et al., 2005). This hypothesis merits further attention. Also, an interesting conclusion drawn from the OSPAR data-sets and one that is relevant here is that, superimposed on the area-wide assessments of impact, there may be significant local variation – attributed, in the case of TBT, to impacts from shipyards, ports and harbour activity. Consequently ecological status may vary from good to bad within a few km or less, depending on currents, proximity to hotspots, and the degree of exposure of water bodies. Spatial scales of impacts are highlighted in other sections of our report and there is strong case for continuing to investigate the causes and extent of small-scale variation in ecological and chemical impacts in coastal ecosystems in the Channel Region.

In conclusion, high sensitivity and specificity of *N.lapillus* to TBT, the ease with which imposex can be determined and quantified (based on RPS, VDS, percentage incidence) and wide geographic distribution throughout the Channel make the dogwhelk an exceptional indicator for this form of androgenic endocrine disruption. Observation of population composition and abundance also allows an appraisal of reproductive competency. When combined with appropriate analytical chemistry, these measures provide a reliable expression of TBT impact, and equally important, of the effectiveness of management responses to this threat.

There has been a marked recovery in TBT pollution at many sites, which is reflected in lower levels throughout the region, compared with concentrations at the height of TBT inputs. Reduction in imposex is still ongoing more than two decades after TBT restrictions were first implemented (timescales commensurate with those described across the Channel, in Brittany, by Huet et al 2004).The decrease in imposex at sites in SW England is especially notable at the open coastal sites in North Cornwall.

Overall, recovery of *N.lapillus* populations is occurring, commensurate with the decline in TBT. This is an important finding in that it adds to the weight of evidence (if it were needed) linking the imposex phenomenon with TBT. This unique association has previously been questioned, and imposex attributed to other causes. The results presented here - of the correlation between declining imposex and TBT levels - would seem to dispel any doubts as to the involvement of TBT in reproductive impairment in molluscs, thus vindicating original hypotheses over cause and effect.

However the rates of recovery may vary considerably at different sites and we still need further long-term observations to understand the conditions where recovery is impeded. Even on the exposed coastline of North Cornwall recovery of populations can vary at adjacent sites illustrated by imposex trends at Bude and Widemouth. An algal-bloom-kill at Bude in 1995 was considered as a one possible influence confounding overall recovery rates from TBT pollution (Gibbs et al (1999).

Despite the relatively rapid general improvement of *N. lapillus* populations following TBT legislation, imposex is still present at a large number of sites, albeit at low levels, and there are still some contaminated locations where recovery is not marked. For example, dogwhelks were abundant throughout much of the Fal estuary in the 1970's but were eliminated by TBT in the early 1980's and remain absent: Upstream of Castle Drive, there was no evidence of recolonisation in 2012 and it may be a considerable time before recovery is considered successful. Almost certainly this is linked at least in part, to TBT persistence in sediments and re-release during dredging and similar maintenance operations and disposal, together with continuing low-level residual input from dockyards and marinas (Langston et al., 2003; Langston and Burt, 2007).

The result is a gradient of TBT impacts in the Fal estuarine system with recovery inhibited most significantly at the siltier upstream regions and greatest improvements at sites near the mouth of the estuarine system –correlating with the cline in organotin concentrations. Given the persistence of TBT in sediment, further research and surveillance into the release of TBT from particulates and the impact on *N.lapillus* and other sensitive molluscs is seen as a priority (for example in the context of providing improved TBT action levels for dredged materials).

Situations such as the Fal highlight the need to study variation in spatial and temporal scales of ecological recovery. As such they provide valuable lessons for management of future marine pollution scenarios whether it be TBT or emerging pollutants.

The results for *Nucella* are encouraging, for the most part, in highlighting the improvement in TBT levels and effects, following legislation. More extensive monitoring would be valuable to confirm spatial and temporal trends across the entire Channel area. At present, the indications are that it may take at least a quarter of a century for some moderately TBT-polluted estuarine locations to recuperate from dockyard and port activities: persistent sediment loadings are likely to delay recovery even longer in areas such as the Fal. It would be useful to examine similar sites within the Channel Region to gain a broader view of the influence of the EU legislation on recovery of TBT-impacted ecosystems. Given that immature female dogwhelks are more sensitive to short term changes in TBT compared with mature females, monitoring using immature cohorts may provide the best assessments of improvement (or deterioration) in water quality in future surveys of imposex.

In summary, the work on *N. lapillus* populations in SW England, undertaken as part of the DIESE project, has highlighted the value of dogwelks as sensitive, reliable and relatively simple bioindicators, with widespread relevance for monitoring the masculinising impacts of TBT in the Channel and further afield. Such outputs will be relevant in the context of producing future Quality Status Reports for the region.

IV.8.3. Intersex, recovery from TBT and contaminants in clams Scrobicularia plana and sediments in the Channel

Various natural and man-made compounds in domestic and industrial effluents are capable of altering reproductive processes in wildlife. Endocrine disruption, manifested as feminisation of male fish in rivers, caused by (xeno)oestrogens, is well documented (Tyler et al., 1998) although the extent of effects in invertebrates, particularly marine taxa, is relatively unknown. We present evidence of widespread feminisation in the estuarine clam *Scrobicularia plana* throughout the Channel region, which may be linked to urban, industrial and agricultural influences.

Given the current debate over the presence of endocrine disruption in the marine environment, the question of whether anthropogenic forces can influence the sexuality and reproduction of aquatic invertebrates is particularly important, as invertebrates comprise 95% of all animal species and are central to ecosystem function (Defur et al., 1999).

The sexual identity of bivalve molluscs ranges from strict gonochorism (separate sexes) to functional hermaphroditism (Delgado & Camacho, 2002). There is also a suggestion that some bivalves may switch readily between states (Mackie, 1987).

Populations of the common estuarine bivalve *Scrobicularia plana* have previously been reported to be inherently gonochoristic but may display intersexuality under certain conditions thereby acting as potential indicators of endocrine disruption (Hughes, 1971; Rodriguez-Rua et al., 2003; Sola,

1997). In populations of *S. plana* in south west UK, we have shown that gonad differentiation becomes evident during June, and by early July the majority of adults have developed gonadal cells, which in the majority of individuals, are either sperm or oocytes. In an intensive survey of a population from the Avon Estuary, South Devon, however, we found that a number of the male gonads contain oocytes, in addition to sperm cells (ovotestis or intersex) (Langston et al., 2007).

We have since examined populations of *S. plana* in other estuaries and discovered varying degrees in the 'severity' of intersex of males, i.e. a single oocyte, clusters of oocytes, whole follicles packed with oocytes, and in extreme cases, gonads dominated b 'female' follicles. Whilst the proportion of females in these populations remained unaffected (50%), males exhibited varying degrees of feminisation. Furthermore this condition appears inducible by some known endocrine disrupting chemicals (Langston et al., 2007).

Based on these observations and methods we have set out to examine the severity and frequency of intersex in *S.plana* throughout the channel region throughout the four years of the DIESE programme.

A key objective is to evaluate the long-term effects of anthropogenic activity on the sexuality and reproductive output of clams. In addition to the above feminisation phenomenon – we have included some work on the anticipated recovery of *S.plana* in TBT-impacted ecosystems (Poole and Southampton Water).

Using updated time-series on chemical and biological indicators we have established the prognosis for *S.plana* at sites where sediments are a persistent source of TBT and are thought to have been responsible for population decline in *S.plana*. Studies have included: an up-to date assessment of TBT pollution in Poole Harbour and Southampton Water (impacted by the leisure fleet and/or commercial shipping) and an evaluation of progress towards recovery following national and global regulation on TBT in antifouling paints. The DIESE project has provided important information on the effectiveness and timescales of pollution control of this well-known contaminant of marine habitats, as well as developing protocols for visualizing impacts from other, new -emerging pollutant threats such as (xeno)oestrogenic/anti-androgenic compounds.

Thus, the methodology for assessment of clam populations, originally designed to study TBT impact was applied to the *study of feminising effects* in *S. plana* throughout the Channel Region (where we have observed populations of *S. plana* which are affected by intersex).

During the first year of the project (2009) the MBA group developed a field sampling campaign for DIESE, which began with trips to the Avon (Devon) Poole Harbour, the Severn Estuary and Southampton Water. In total 19 sites were sampled for clams, sediments and a range of environmental parameters (Fig 72 & 73). These included various sediment parameters such as grain size (laser particle sizing), organic matter determination (weight loss on ignition at 400°C), organotins (TBT and DBT) a range of metals (both anthropogenic and geogenic to help apportion relative contributions from these sources – a surrogate measure of pollution impact). These methods are broadly based on those described in previous studies (Langston et al., 1999; Ridgway et al., 2003). Sub-samples (sediment and clams) were also distributed to DIESE project partners for additional chemical and molecular characterisation. As the effects on gender caused by (xeno)estrogens and/or anti-androgens may have similar repercussions for the viability of stable populations to those demonstrated for TBT, our estuarine surveys have combined measurement of effects on individuals (intersex frequency, severity and sex ratios) with higher-level consequences (population composition) and we have attempted to explore their connections with environmental chemistry.

Frequency and severity of Intersex, measured by examining gonad histology by light microscopy are based on techniques described previously (Langston et al., 2007), using a sample of 30 clams at each site. Sex ratios are also determined (Fisher exact test, $P<0.005$). To complete the DIESE field programme, populations of clams and other benthic macrofauna were sampled semi-quantitatively at the majority of estuarine survey sites - to establish linkages between higher-level biological responses (abundance of juvenile and adult clams, perturbations at the histological level (intersex), and contamination levels.

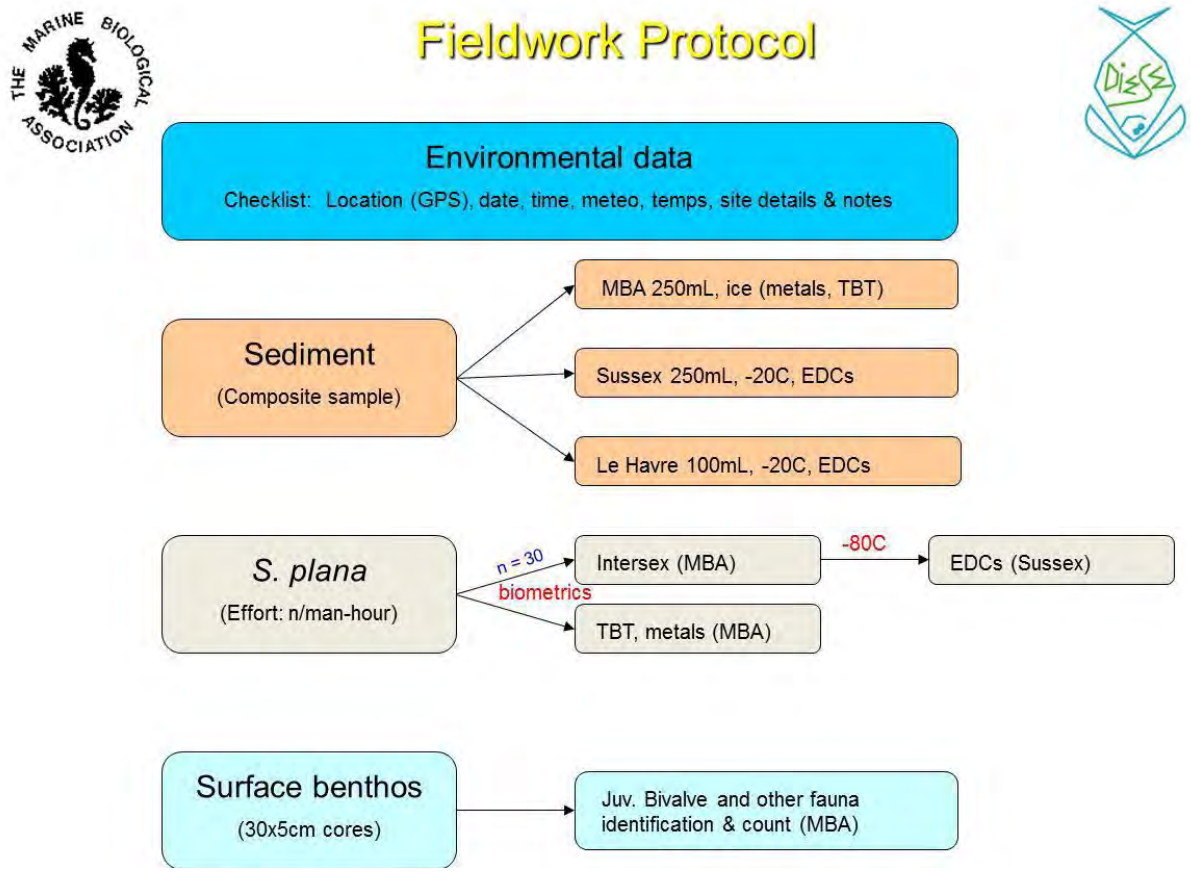


Figure 72: Estuarine fieldwork programme undertaken for DIESE

Sampling of adult clams at each site (for intersex determination) was timed to give a semi-quantitative measure of abundance. Alongside sampling of *S. plana* and surface sediment, additional sampling of surface benthos was undertaken at most estuarine sites visited. The objective being that the data generated from these samples will help provide insight into the benthic community structure at each site and establish any linkages between these communities and sediment chemistry. The data will help determine any influence of pollutants, including endocrine disrupting chemicals (EDC 's), on clam populations and possibly other macrofauna.

Populations of juvenile clams and other surface benthic fauna were sampled from 30 cores (6.4 cm diameter and 2cm depth). Cores at each site were pooled (total area 0.0956m2) and stored in air filled bags in a cool box until their return to the lab, where they were transferred to a wooden box sieve (1mm mesh) and washed gently with seawater. Samples were fixed with 4% formalin. Rose Bengal, was added to the fixed samples to aid sorting.

Prior to processing, formalin was removed. In a fume cupboard samples were emptied onto a 1mm mesh sieve and washed with freshwater to remove the formalin solution (a formaldehyde meter was used to check levels were < 1ppm). Samples were sorted on a shallow tray and transferred to a suitable vial, one for each faunal group. Alcohol (70% ethanol/IMS) was added to each vial to preserve specimens prior to identification. Individual specimens were identified down to species level where possible, using standard taxonomic keys, and counted. Size was also recorded for all bivalves). Each species, once identified was transferred to a vial (one vial per species) containing alcohol preservative (70% ethanol/IMS). Each vial was then labelled with species name, site location and sampling date. Tally counters were used where common species were numerous and easy to identify. In some cases where specimens were damaged, assigning a species name was not possible and in these circumstances the lowest possible taxonomic level was recorded. Furthermore, as many specimen parts could be found in one single sample, only individuals with an anterior end were counted. Differentiation was also made between adult and juvenile specimens where appropriate.

Multivariate analysis of the final data set will compare similarities between sites (intersex, clam numbers, and general macofaunal abundance) with environmental factors.

During 2010, surveys of sediments and clams were extended to the Central and Eastern Channel Region between Selsey Bill and the Medway, namely at Bosham, Dell Quay, Pagham (2 sites), Littlehampton, Shoreham (2 sites), Newhaven, Rye, Ramsgate and The Swale and Medway Estuaries (5 sites). Further sampling was carried out in the south west estuaries - in the Fal (4 sites), Fowey (3 sites), Bristol Channel (1 site), Yealm (3 sites), Axe (2 sites), Otter (1site), Exe (3 sites) and Teign (2 sites). (see Figure 73).

Figure 73: All MBA sampling sites (71UK, 37 French). Samples showing intersex in *Scrobicularia plana* are depicted by red stars

During 2011 further sites were sampled in the south of England in the Plym Estuary (2), the Tamar (3), Lynher (3), Camel Estuary (3), Thames Estuary (7) and West Looe. Also in 2011 we began to include samples from the French Channel coast (see Figure xxx) at Porsgwen, Hotellerie, Cap Hornu, Berk, Etaples, Saint Jacut, Saint Benoit (see Figure 9).

In 2012, the estuarine sampling campaign for DIESE, was expanded and focused on sites along the French coast (from Normandie in the East to Brittany in the west). Several sites in SW England were also sampled (see Figure 9). Colleagues from Universite du Havre helped to provide samples along parts of the French coast. In total, 30 French sites were sampled for clams, sediments and a range of environmental parameters. Again sub-samples were archived for distribution to DIESE project partners for additional chemical and molecular characterisation. To test whether effects on gender caused by EDC may have repercussions for the viability of stable populations, sampling at a

number of sites combined measurement of effects on individuals (intersex frequency, severity and sex ratios) with sampling to evaluate higher-level consequences (population and community composition). These data will also be used in multivariate tests to explore their connections with environmental chemistry.

Exposure trials/ transplants with clams subjected to model anti-androgens (Irgasan = triclosan) have been used to investigate possible causes and mechanisms of ED more rigorously - to evaluate if exposure of *S.plana* to anti-androgens stimulates development of the intersex condition. Experimental exposures with *S.plana* were of two basic types: simple aqueous exposures and exposures to spiked sediments for one month early in gametogenesis (Feb-March), followed by transplant of clams back to the field where they were subsequently recovered on maturation in July. Gonadal development was determined in recovered clams and compared with that in native clams and experimental controls. Endpoints measured in these experiments include sex ratios, intersex frequency and severity, and oocyte diameter. Tissues of *S.plana* exposed to Irgasan (anti-androgen) were also subjected to molecular investigations (U. Sussex) to see if there are associated changes at the molecular level which could be used as markers.

The MBA team has collaborated extensively with researchers at University of Sussex, and Universite du Havre, throughout the project, to investigate the intersexuality phenomenon in clams within the Channel region, and to try and establish causes and mechanisms.

Recovery of clam populations from TBT:

Scrobicularia plana has proved an excellent model to show the extent and timescales of impact and recovery from TBT pollution in sediments. This is depicted in the two examples below, from Southampton water. In the Hamble Estuary (dominated by leisure vessels) clam populations were impacted during the 1980s until legislation on small boats let to a reduction in TBT residues. In 2009 at the start of the DIESE project we established that clam numbers had returned to values similar to those two decades previously (Fig. 74)

In the Test Estuary recovery appears to have been slower due to the predominance of commercial vessels and docks (Fig. 75). Legislation on this sector was not fully effective until the IMO global restrictions in 2008. This is reflected in delayed reduction in TBT concentration and corresponding erratic recruitment of clams. It remains to be seen if this recovery is sustainable.

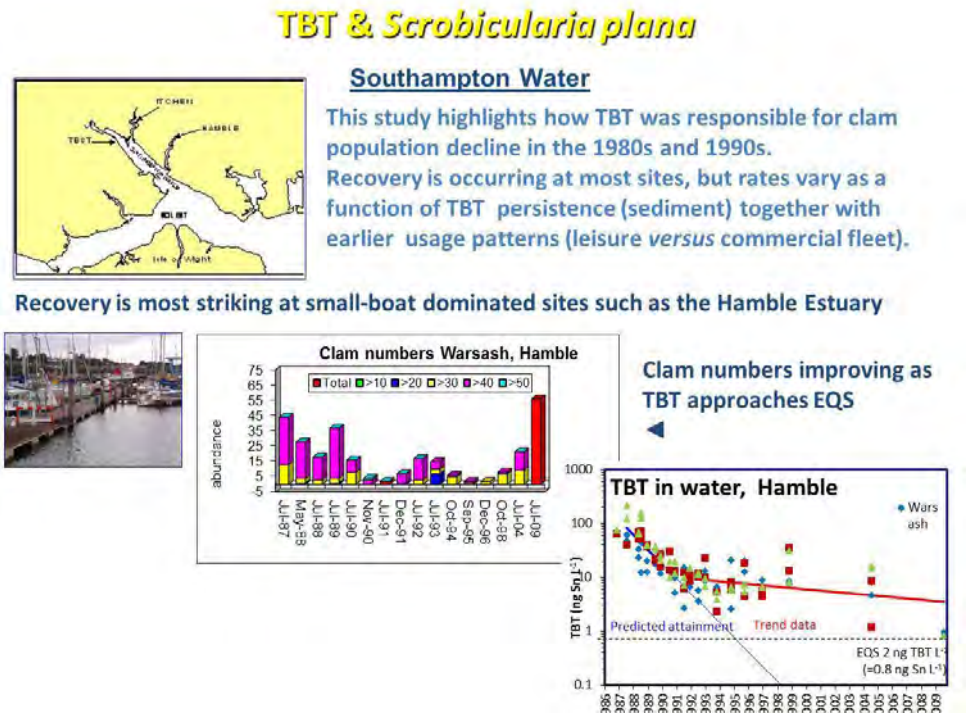


Figure 74: Hamble Estuary (Warsash) showing recovery of clams *Scrobicularia plana*, in response to the reduction in TBT levels following legislation on the use of small boats in 1987.

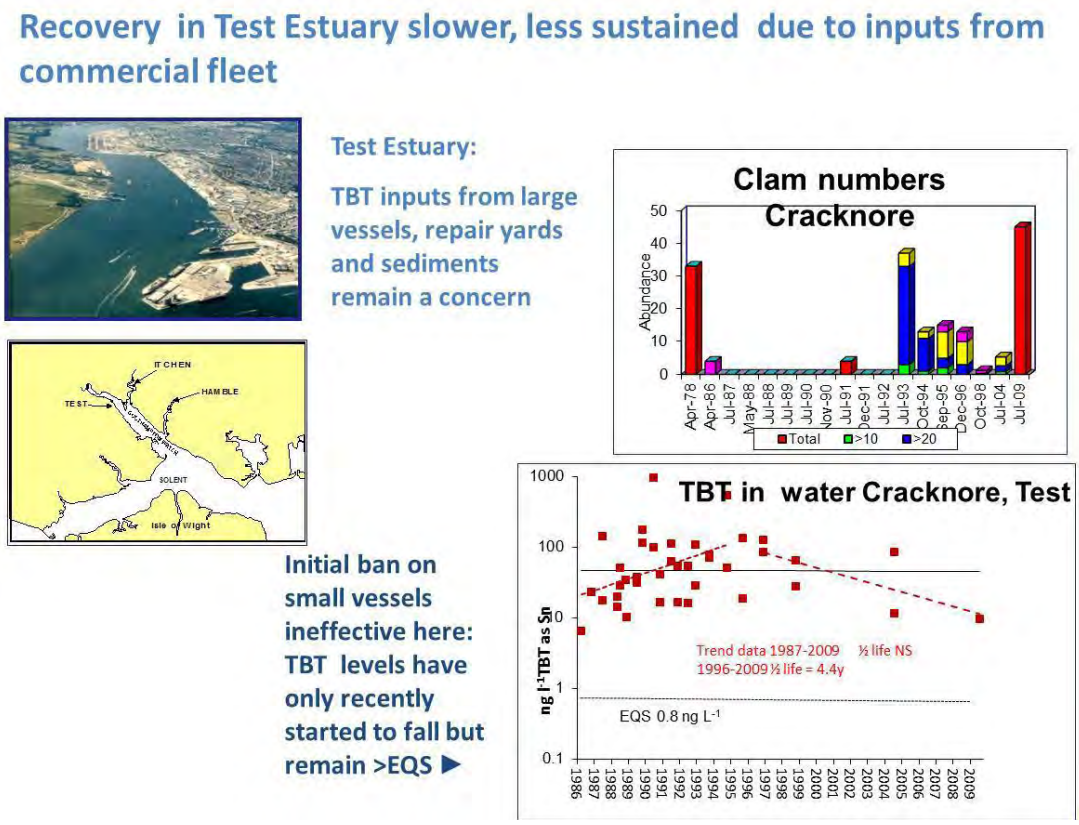


Figure 75: Test Estuary (Cracknore) showing erratic recovery of clams *Scrobicularia plana*, in response to the slow reduction in TBT levels following legislation.

Intersex in *Scrobicularia plana:*

Although intersex was not found at every site, few estuaries have completely gonochoristic *S. plana* populations. Thus, in the Tamar Estuary, near Plymouth, for example, the incidence of intersex ranged from 0% to 25% (mean 9%).

Of the sites sampled in 2009 the Severn Estuary displayed raised levels of intersex and two populations were skewed towards females (figure 76). The Severn is highly industrialised and urbanised, and has been subjected to raised levels of metal contamination and other impacts[11]. The incidence of intersex in *S. plana* from sites such as the Avon (Devon) is unlikely to be due to industrialisation since the catchment area is predominantly rural; however, sewage and agricultural inputs are possible sources of oestrogenic compounds and could be a factor in increased intersex levels in a number of apparently pristine locations throughout the region. It is important to determine the mechanisms and causes in greater detail in future studies.

Initial chemistry data for the 2009 samples indicated high levels of anti-androgenic activity at a number of sites (see Chapter Two, this report), suggesting widespread contamination of chemicals that have the *potential* to feminise clams. However, preliminary observations of distributions of anti-androgen contamination and the incidence of intersexuality in *S.plana*, are not indicative of a strong cause-effect relationship. Likewise exposure trials with model anti-androgens to investigate causes and mechanisms of ED failed to reveal strong feminising effects in clam gonadal tissue. It is not inconceivable however that the slight effects observed in some experiments could be enhanced in the presence of oestrogenic compounds.

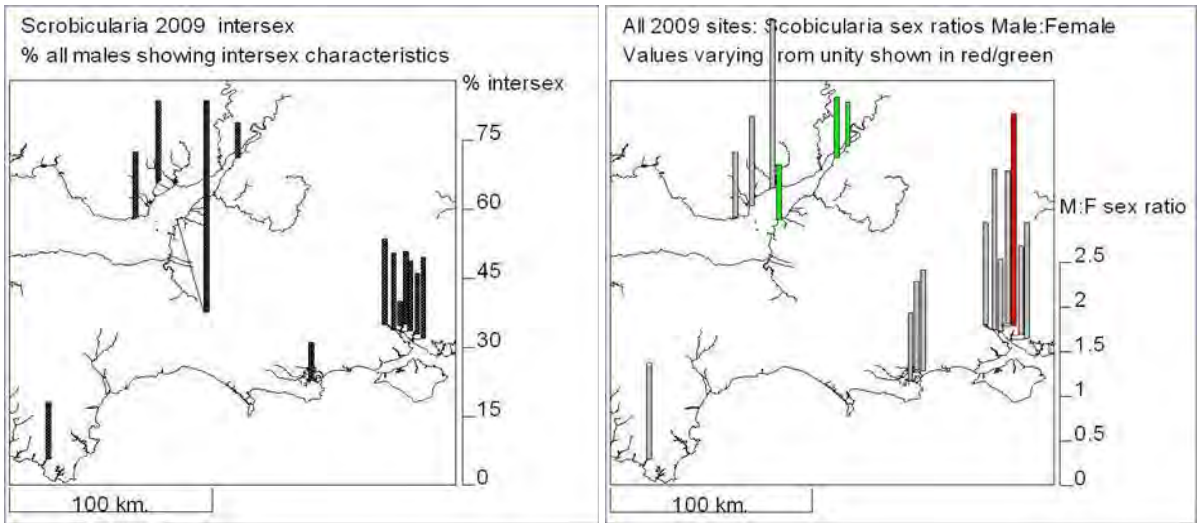


Figure 76: *Scrobicularia plana sex* ratios showing two populations in the Severn Estuary skewed towards females and one in Southampton skewed towards males.

Intersex was present in 12 out of 32 populations sampled in 2010, albeit at low levels, mostly (Fig. 77). Highest intersex frequencies (10-20 % of males affected) were found at Pagham and in the Yealm (which also had examples of males with a high intersex severity index). *S. plana* were free of intersex at more than half the sites examined and this condition appears to represent 'baseline' (Blue flags, Fig. 77). The sex ratio was skewed significantly from the normal 1:1 M:F distribution at a small number of sites. Sediments and clams have been analysed for organotins and metals and sub-samples have been characterised for oestrogenic and anti-androgenic activity by partners at Sussex.

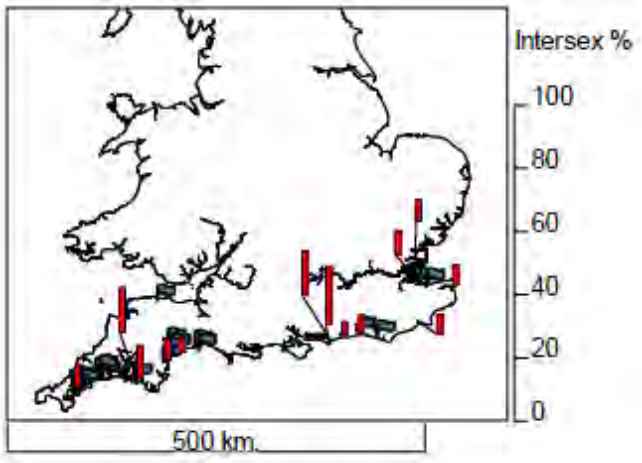


Figure 77: Sampling sites and incidence of intersex in clams *Scrobicularia plana*, 2010.

2011 samples included French sites two of which (Berk and Etaples) exhibited around 50% intersex. Further west intersex levels were mainly 10% or lower. At St Benoit however the population was significantly biased towards females, whilst at Porsgwen males exceeded females by a factor of 2:1

2012 samples saw further sampling of French sites from Normandie to Brittany (n=30). Several sites in SW England were also sampled.

On completion of the final analyses across all sites, intersex was present in approximately 70% of the 37 *S. plana* populations collected at French sites, and 52% of the 71 UK Channel sites . The sex ratios showed a few sites with biased populations (six populations skewed towards males, seven towards females) though the mean m:f ratio was close to unity (1.04) for UK samples, and marginally biased toward females (0.917) in French samples. Impacts were quite similar on both sides of the channel.

A survey of temporal trends has been continued in the Avon estuary in SW England where intersex levels had previously been considered high, potentially due to inputs the influence of cattle in the catchment and adjacent fields (note pregnant cows excrete large amounts of oestrogens). Intersex levels here have declined in recent years, and may signify an improvement in water quality possibly related to improved agricultural practice (fencing of cattle from the estuary) coupled with an overall decline in the national dairy herd. Clearly although agriculture is a potential source of feminisation, there may confounding features influencing cause and effect which we have yet to determine.

In laboratory experiments conducted previously, we have observed an increase in intersex in *S. plana* following exposure to mixtures of oestrogens (17β estradiol) and xeno-oestrogens (nonylphenol) (Fisher exact test, $P<0.05$) (Langston et al., 2007). In experiments during DIESE, BPA (bisphenol A) has been somewhat effective, as has Irgasan (Triclosan) though the latter compound has shown a slight alteration in sex ratio (towards females) rather than a higher increase in the incidence of intersex.

Benthic sampling

Examples of benthic sampling outputs are shown below. Total adult clam numbers collected per man hour effort are shown in Fig 78A. Low numbers (in red) were a feature at some sites in the Severn Estuary. This coincides with areas showing high intersex though it is not possible to establish cause and effect at this stage. Juvenile *S. plana* were present in small numbers in five of the core samples taken in 2009 (Fig. 78B) but were not identified at the majority of sites, including those in the Severn Estuary.

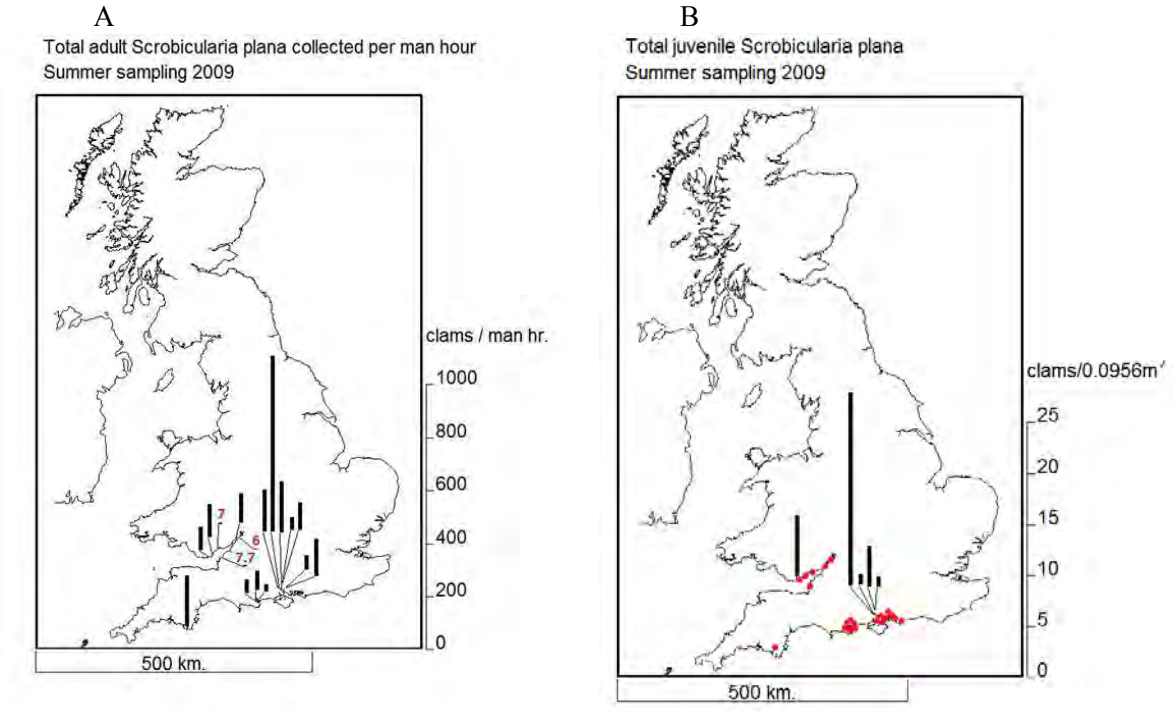


Figure 78: Adult *Scrobicularia plana* per man hour (A) and juvenile abundances in cores (B) in sites sampled in 2009.

Comparisons of bivalve numbers show that juvenile *S.plana* are in fact outnumbered by other species. *Macoma balthica* and *Abra tenuis* tend to dominate In the Severn and Poole/Southampton, respectively though again it is not possible to establish cause and effect at this stage.

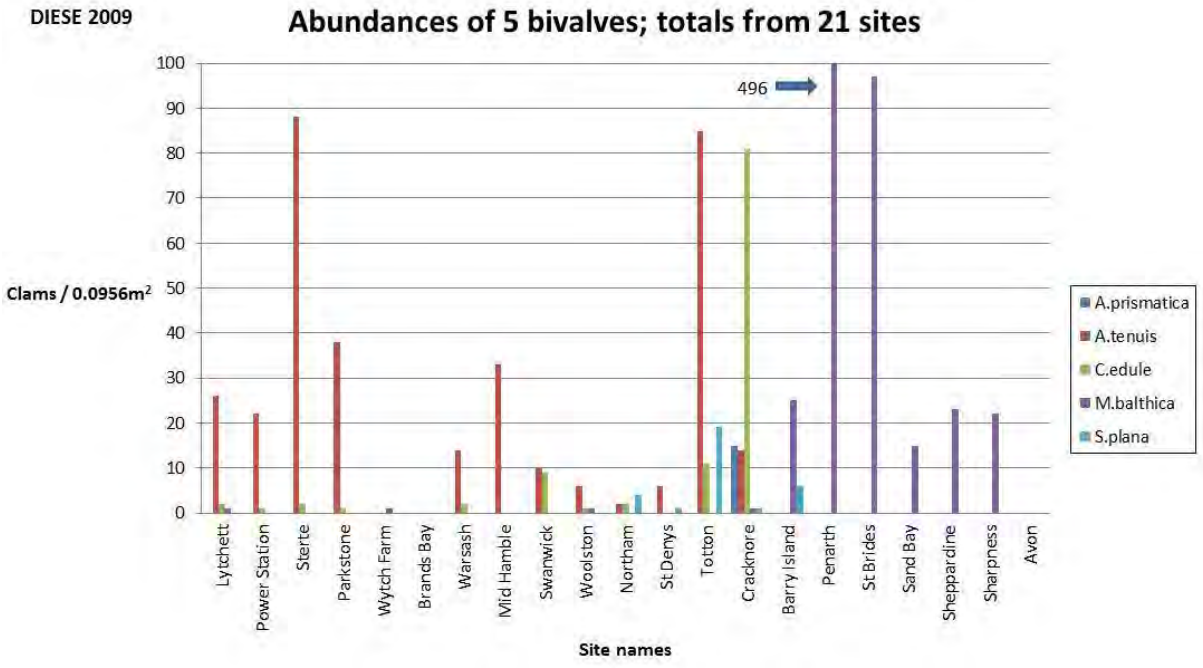


Figure 79: Small bivalve abundances from Intertidal core samples 2009.

Cluster analysis of macrofaunal assemblages is used to compare similarities and dissimilarities between sites (Fig. 80). This too identifies many of the Severn sites as being distinctive from many other Channel sites. There is a requirement to match macofaumal distributions with environmental determinants to identify causes.

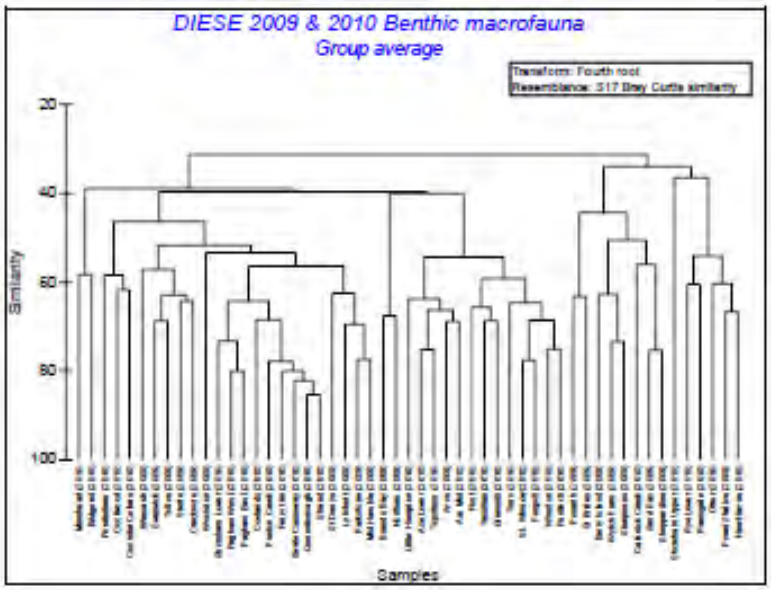


Figure 80: Cluster analysis of macrofaunal assemblages 2009, 2010.

Chemical determinants show some distinctive distributions across Channel sites as exemplified by Cu concentrations in sediments in fig 17. This plot indicates Cu values (µg g-1dw) in sediment samples in relation to Interim Sediment Quality Guidelines (ISQG for Cu = 18.7 µg g-1) proposed by CCME (1999). The ISQG equates to Threshold Effects Levels (TEL)– concentrations below which biological effects are unlikely (baselines). Also shown are Probable Effects Levels (PEL=108 µg Cu g-1), above which, effects might be expected.

Several sites in mineralised areas of SW England, Southampton (Cu-based antifouling) and the Thames region exceeded TEL and also the PEL in the SW region and Southampton (effects likely).

None of the French sediment samples exceeded PEL. Indeed most of the French samples were <TEL for Cu. It should be noted however that this may reflect granulometry rather than (absence of) contamination. Many of the French sediments had a larger grain size composition (sandier) than the finer silts representative of UK estuarine sites sampled and therefore would be expected to be lower in anthropogenic metals, irrespective of inputs.

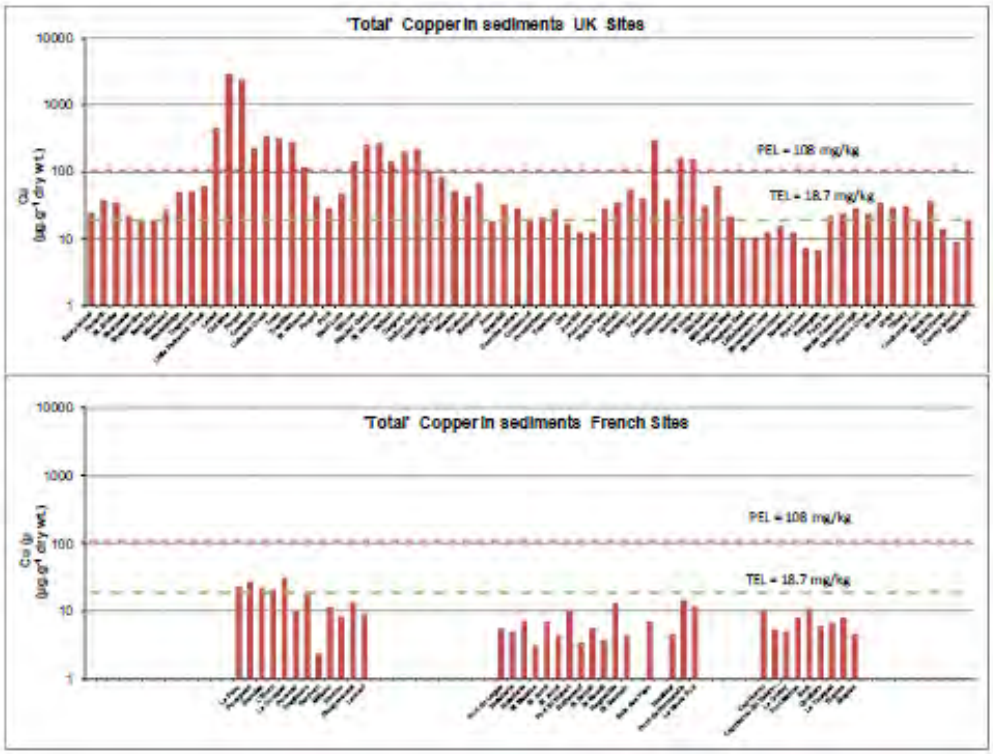


Figure 81: Cu concentrations (µg g-1dw) in sediment samples in relation to Probable Effects Levels (PEL) and Threshold Effects Levels (TEL) (see text for explanation).

In conclusion, intersex occurs widely in the clam *Scrobicularia plana* on both sides of the Channel and appears to be an excellent indicator species to study biological responses to endocrine disrupters and other chemicals. Whilst intersex is mostly at a low level (10% of males affected, or less) there are sites which are clearly above background (50% of males affected) and some populations where sex ratios are skewed (sometimes towards females, sometimes males), most likely as a result of anthropogenic influence. Thus, whilst at present the causes of the intersex condition in nature remain speculative there is enough evidence from our laboratory and field observations to indicate that disruption from the perceived gonochoristic sexuality of *S. plana* is occurring widely throughout the region and is inducible by known endocrine disruptors. Further studies are needed to define, more adequately, true baselines, the link between sexuality of these bivalves and anthropogenic causes, and the fine detail of the extent of impacts in relation to catchments.

High levels of intersex in the Severn estuary, UK, are accompanied by low abundance of *S. plana*, and somewhat unusual macrofaunal composition. However more work is needed to confirm the links between endocrine disruption (as signified by intersex frequency), fecundity, population density and community/ecosystem consequences

IV.9. Investigation on mutagenic compounds in sediments

IV.9.1. Introduction

Many natural or synthetic compounds are potential or known carcinogens and identified as such in the European regulation (CLP 1272 / 2008). Among them, polycyclic aromatic hydrocarbons (PAHs), hexachlorobenzene, hexachlorocyclohexane (HCH), methyl mercury and cadmium appear on the list of the eleven priority hazardous substances of the European Water Framework Directive (Directive 2000/60/EC). Because of their widespread use and / or physicochemical stability, these compounds are present in significant quantities in most major highly urbanized and industrialized European and North American estuaries (Budzinski et al 1998; Yunker , et al 1999).

Mutagenic or genotoxic potential of certain molecules can be expressed directly, i.e. without any metabolising steps, when interacting with biological molecules in the body, or indirectly after "bioactivation". This bioactivation is derived from the action of cellular enzymes that metabolise the molecule so it can be more easily excreted. However, in case of bioactivation, the transformation generates a highly reactive and potentially mutagenic compound. Thus a metabolite more dangerous than the parent compound is produced. The objective of this work was to evaluate the mutagenic potential of sediment extracts collected along the French and British coasts through the use of the SOS Chromotest.

The SOS Chromotest is an in vitro test used to evaluate the genotoxic activity of a pure substance or a biological or environmental sample. This test uses a bacterial strain, *Escherichia coli* PQ37 to determine the ability of a chemical to induce DNA damage by quantifying the expression of a component of the SOS repair system, the sfiA gene (Quillardet et al , 1982). In this bacterial strain, the lacZ gene, responsible for the synthesis of β -galactosidase is under the control of the sfiA promoter. Thus, when the bacterial DNA is damaged by genotoxic compounds, the SOS repair system is activated, and leads to the induction of the lacZ gene and the synthesis of β-gal enzyme whose activity is quantified by optical density (the appearance of a yellow color) at 405 nm. The activity of the β-gal is compared to the activity of alkaline phosphatase (ALP) measured in parallel, which constitutes an internal standard as it is not inducible by genotoxic agents. To detect indirect genotoxic compounds, a metabolic activation is produced by adding a S9 fraction containing metabolic enzymes (including cytochrome P450) to the culture medium. The genotoxic activity for a given sample concentration (c) is expressed by the ratio Rc = β-gal/ALP. The induction factor, IF, is defined by the ratio Rc/Ro where Ro is the ratio measured for a negative control (solvent alone). A sample is generally considered genotoxic if IF is greater than or equal to 2, moderately genotoxic if $1.5 \leq FI < 2$ and non-genotoxic if $IF < 1.5$.

IV.9.2. Results

Organic sediment extracts were produced by solvent assisted extraction (ASE) and used for the SOS Chromotest. The results show that in the absence of activation by the S9 fraction, direct genotoxic are detectable in sediments from the French and British coasts (Fig. 82). Similarly, after bioactivation, some sites show the presence of indirect mutagenic compounds (Fig. 83).

All results are plotted on maps of the Channel coast (Fig. 84).

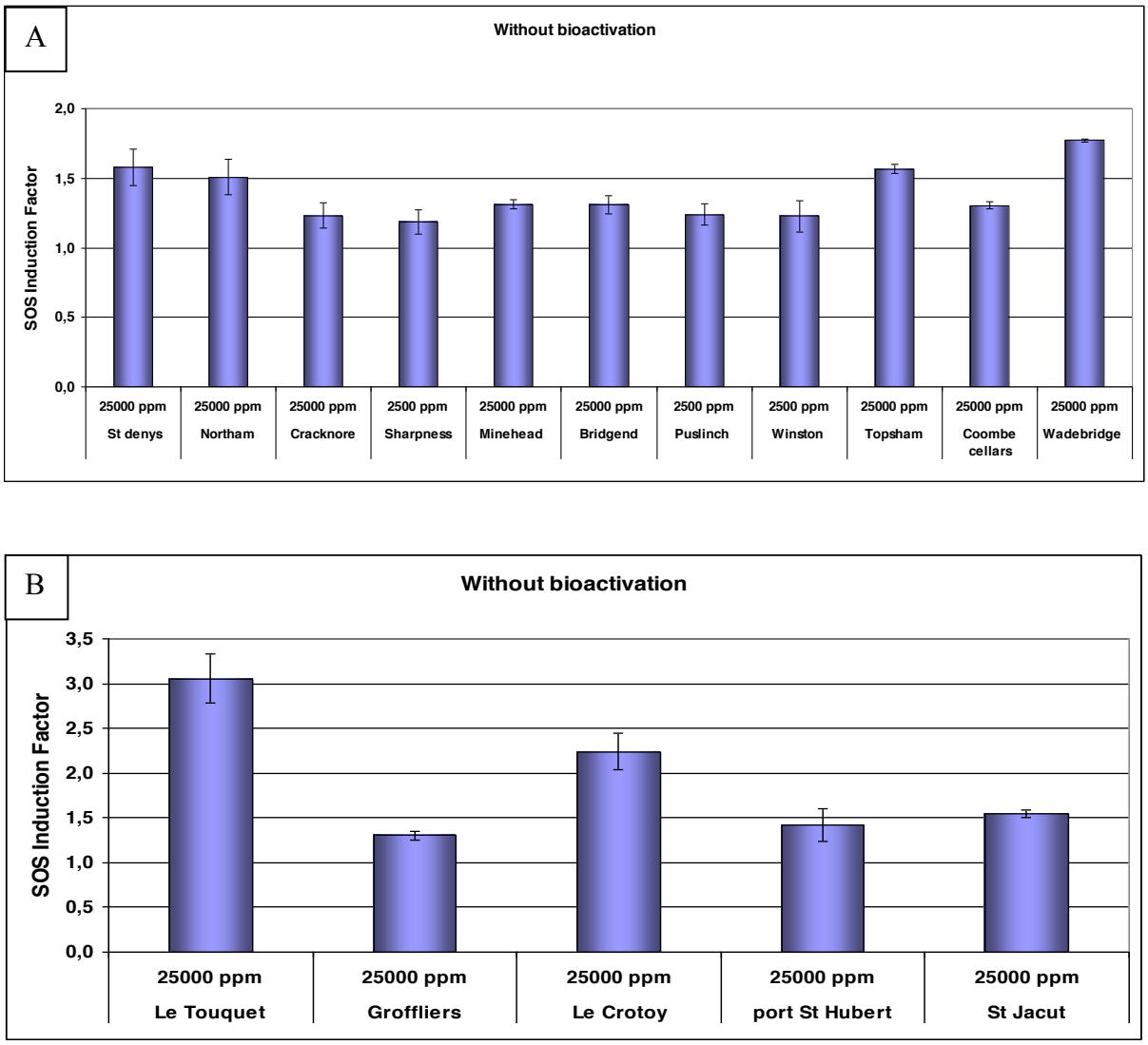


Figure 82 : Induction factor (IF) of the SOS Chromotest obtained with sediment extracts from British (A) and French (B) coasts without bioactivation. A sample is generally considered genotoxic if IF is greater than or equal to 2, moderately genotoxic if $1.5 \leq FI < 2$ and non-genotoxic if $IF < 1.5$.

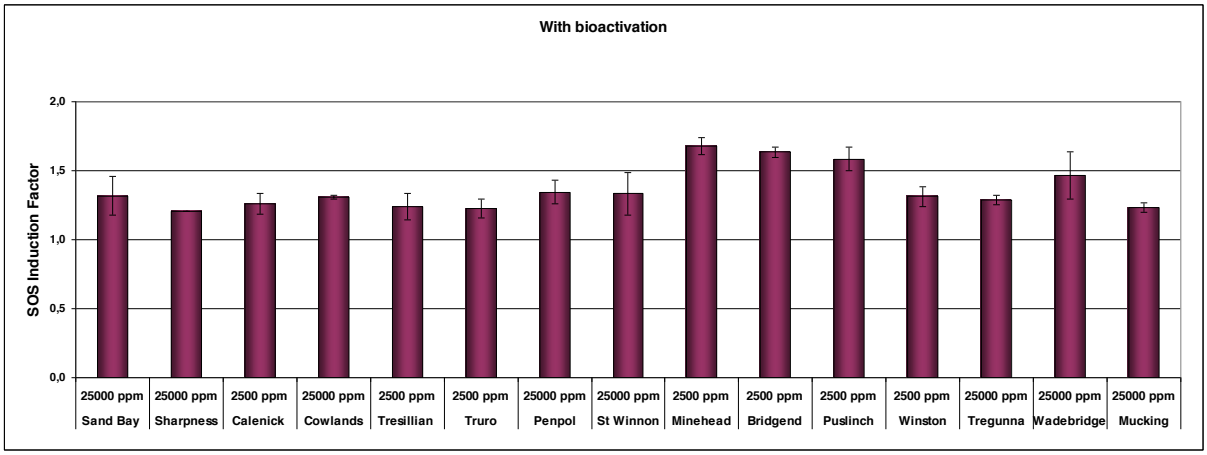


Figure 83 : Induction factor (IF) of the SOS Chromotest obtained with sediment extracts from British (A) and French (B) coasts with S9 bioactivation.. A sample is generally considered genotoxic if IF is greater than or equal to 2, moderately genotoxic if $1.5 \leq FI < 2$ and non-genotoxic if $IF < 1.5$.

Figure 84 : Map of mutagenic sediments as detected using the SOS Chromotest on extracts from samples of the British and French coasts. Orange, red and purple dots are indicating the presence of mutagenic compounds (in a growing order) while sites flagged with yellow dots are devoid of detectable mutagenic compounds.

V. CONCLUSIONS

Previous work performed by the DIESE project partners revealed dysfunctions and harmful effects of pollution on the health of organisms, particularly affecting reproduction and development of fish (Minier et al, 2000a and 2000b; Martin-Skilton et al, 2006; Hinfray et al, 2009; Minier and Amara, 2009) or mollusc reproduction (Langston et al, 2007; Peck et al, 2007). These observations show that the aquatic environment, especially in the Manche region, contains substances that can affect living organisms and that the environmental management system and current practices fail to prevent serious effects which wild organisms are experiencing. Thus the work undertaken during the DIESE research program aimed at identifying compounds showing activity deleterious for living animals, at elucidating the mechanisms of action of such xenobiotics present in the environment and at implementing various tools to help the management of aquatic environments.

Identification of active compounds

The first part of the DIESE program allowed to develop or to use biological tools to identify organic compounds present in the environment that are actually responsible for the effects measured. This strategy highlights the biological effects that can guide the search for active compounds. Although it cannot be claimed that an exhaustive identification is feasible, this strategy offers an alternative to the impossibility of measuring all compounds present in the environment. Indeed, chemical compounds are widespread and numerous (approximately 100,000 of common use) and it is likely that only a part of them or a mixture of several compounds are responsible for significant effects. The identification of the mechanism of action of pollutants and the use of measures related to these mechanisms allow to relate the effects it would be desirable to avoid to the presence of disrupting compounds in environmental samples.

In this strategy, the choice of the measured biological effects is important. It is not possible to measure all the effects and only few mechanisms can be implemented. To direct the research, *in vitro* tests were used or developed. It is quick and simple cell-based assays that allow high-through-put study of environmental samples. Targets that have been used in the DIESE program are sex steroid receptors. Indeed, these receptors are very important regulation nodes in the endocrine system and it has been shown that many endocrine disruptors interact with them (UNEP, 2012). Two systems were used: a test using genetically modified yeast that are expressing the human androgen receptor and liver explants expressing the estrogen receptor. The first allows to identify compounds with anti-androgenic activity in the sediments of the Channel coasts while the latter allows to establish a new system specific for fish and able to study the activity of estrogenic compounds.

It has recently been suggested that the feminisation observed in wild fish is due to a combination of estrogenic and anti- androgenic compounds (Jobling et al., 2009). Thus researchers from the DIESE program developed an extraction protocol for compounds present in the sediment to investigate the possible presence of molecules with anti-androgenic activity in environmental samples. The results showed that many compounds such as aromatic hydrocarbons are likely to disturb the physiology of living organisms by interacting with the androgen receptor. Six of them have been identified in sediment extracts. They are the benzanthrone, the fluoranthene, the 1,2-benzodiphenylene sulfide, the benzo [a] pyrene, the benz [a] anthracene , and the 9- phenylcarbazole, some of which showed anti-androgenic potencies similar to that of the flutamide standard. In addition, biocides such as triclosan and chlorophène are also anti-androgenic (Hill et al, 2010; Rostkowski et al, 2011).

Active compounds have been search for in *Scrobiculatia plana* tissues. These clams are showing evidence of dysfunctions of their reproductive. Anti-androgenic compounds have been

detected and their identity is being analysed. Notably, a very active androgen fraction was also discovered in the male tissue. Similarly, work is ongoing to discover the identity of the compounds.

Highlighting mechanisms of action of xenobiotics

In order to increase the knowledge and understanding of problems due to the presence of chemicals in our environment, studies have been conducted on fish and shellfish. These studies have been at the heart of the second part of the program.

Research on shellfish endocrinology poses a particular problem because knowledge is still very fragmented in these organisms. To circumvent this difficulty and also to study in the most comprehensive way the mollusc physiology, "open" procedures involving metabolomics and transcriptomics approaches have been undertaken. Indeed, in the absence of specific knowledge, monitoring thousands of parameters simultaneously may allow to identify pathways and mechanisms that are modified in a particular situation and then lead to more targeted studies in order to develop specific tools.

The metabolomics approach has been implemented on samples of mussels exposed to different doses of the naturel estrogen, 17-β estradiol (E2). Approximately 10,000 signals were measured by mass spectrometry for each sample and some allow to perfectly differentiate exposure conditions. Among the discriminating signals, some compounds have been identified and used to undertake specific experiments and identify mechanisms of action for steroids in molluscs. This was the case of prostaglandins and amines that were differentially expressed in the presence and absence of estradiol and led to study the role of amines (5-HT) and enzymes involved in the synthesis of prostaglandins (COX) in mollusc physiology. From these data, we examined the responses of 5-HT receptor and the expression of COX gene in mussels, *Mytilus edulis*, exposed to estrogenic compounds during different stages of their reproductive cycle. The results showed that the expression of 5-HT receptors and COX enzymes levels are different in males and females, indicating a specific role for different gender and confirming the involvement of estrogens in mollusc physiology. In addition, the results showed that environmentally relevant concentrations of the natural estrogen, E2, and of the synthetic estrogen, EE2, may induce changes in the expression levels of 5-HT and / or COX in mussels. In particular, E2 decreases the level of expression of COX enzymes in females while the steroid has no effect on male bivalves. Considering the expression of 5-HT receptor, exposure to E2 resulted in a decreased expression of the gene in mature males and females. In contrast, E2 exposure during early gametogenesis generated a very large increase in receptor expression.

These results indicate that important mollusc endocrine parameters can be modified by exposure to estrogens and thus bring insight on the mode of action of steroids that lead to disruption of the reproductive system sometimes observed in the wild (Blaise al, 2003; Gauthier-Clerc et al, 2006). The occurrence of different results depending on the stage of sexual development during exposure to estrogens confirms the difficulty of understanding the risks associated with endocrine disruptors because the effects do not depend only on the dose but also the exposure time.

To further explore the involvement of steroids in the physiology of molluscs, suppressive subtraction hybridization techniques (SSH) was used on mussels gonads samples from different stages of gametogenesis exposed to estrogens in controlled conditions. Several differentially regulated genes, including the gene associated with the androgen receptor, lysine and the yolk sac of an envelope sequence were identified. These sequences provide new direction for research in order to understand the role of sex steroid hormones in invertebrates and provide a means to identify endocrine disruption in these organisms.

The SSH technique has also been applied to the research and understanding of mechanism of disruption of the *Scrobicularia plana* reproductive system. This bivalve is subject to inappropriate differentiation of male cells within the testes resulting in the formation of oocytes characterizing intersex individuals. Several interesting mRNA transcripts were identified and validated by

quantitative PCR. These transcripts appear repressed in intersex samples. They encode a variety of proteins involved in cell signalling (RACK1), cell cycle regulation (PCNA, histone H3), protein synthesis (ribosomal proteins), the cytoskeleton (tektin), the sperm elaboration (tektin, SPL1) and energy metabolism (CYB, COX-1). In many cases, the expression levels are in intersex individuals more than 10 times lower than that of control males thus suggesting that intersexualisation may be due, in particular, to the suppression of masculinizing genes.

The immune system may be subjected to impairment due to xenobiotic exposure. To investigate potential effects occurring during development, studies have been conducted in juvenile sea bass, *Dicentrarchus labrax*. This generated new information on the development of the sea bass immune system and allows to study the effects of exposure to estrogenic compounds during critical phases. In addition, this study further explored the relationships between the immune and the endocrine system. The results allow to characterize the expression of the estrogen receptors α and β2 and of several cytokines in the anterior kidney, the homologue of the mammalian bone marrow and the place of hematopoiesis in teleosts. This organ is particularly important in the immune system because it allows the development and proliferation of B lymphocytes. It is also responsible for developing capabilities of phagocytosis and antigen recognition (Rauta et al., 2012; Agius, 2003). A particular period of maturation, close to 180 days post-hatching, has been identified. A sum of crucial events for the development of the immune system seems to occur in this period of development. We observed peaks of expression of the estrogen receptor β2 and interleukin-1β, a significant migration of B cells out of the anterior kidney, and a very significant increase in the activity of macrophage populations from the anterior kidney. Accordingly, this period, in addition to its obvious importance, could be a window of particular sensitivity of the immune system whose disruption could cause long-term effects. Indeed, repeated exposures of juvenile sea bass to 20 ng E2/L were conducted under controlled conditions during the development of the immune system. Few significant changes in cytokine expression were noted. However clear alterations in the expression of the estrogen receptors and a significant decrease in macrophage activity were measured during the critical period identified, stressing the importance of this period.

To develop a tool to measure potential androgenic or anti-androgenic effects in fish, the bullhead (*Cottus sp*) was studied. Indeed, this species exhibit a significant sexual dimorphism in renal tissue during the breeding season that is androgenic-dependant. However, although this feature has been confirmed and its induction is actually dependant on the presence of active androgens, the measure proved to be not discriminating and further efforts are needed to be able to propose a relevant biomarker of androgenic effect. A proteomic analysis is currently being pursued identify specific androgenic markers.

Research performed on the roach, *Rutilus rutilus*, have identified new alterations in the reproductive system of the fish. While previous studies were tied to study endocrine disruption in male fish, this study has implemented a series of measures to explore the physiology of female roach. The results show that the development of the reproductive organs is compromised in roach living in disturbed areas (such as the Seine River). This translates into a reduction in the relative mass of the gonads accompanied by a developmental delay and a reduction in the synthesis and, consequently, the accumulation of vitellogenin in oocytes. Thus the quantity but also the quality of oocytes is altered in females exposed to a mixture of chemical compounds. It is notable that these alterations appear to be specific the reproductive or the endocrine system because otherwise roach show no alteration in growth or weight and no visible signs of parasitic infestation. Finally, to explore the underlaying mechanism of disruption, the gonadal aromatase activity was studied and the results indicate that this activity is altered in females of the Seine. This alteration may result from the sensitivity of the aromatase to many chemical compounds (Hinfray et al., 2006) and contribute to the hormonal imbalance observed in this species.

Finally, in order to document a suite of biological parameters for flounder (*Platichthys flesus*) and to investigate the ability of a suite of biomarkers to deliver a coherent and informative picture of the health of the fish living on our coasts, measures of DNA damage, molecular and biochemical responses including immunological parameters, isotopic profiles indicators of energy metabolism,

growth and genetic variability were conducted during laboratory exposure experiments or in natural populations of flounder from differentially contaminated estuaries.

Laboratory exposure to a complex mixture of PCBs and PAHs induced several biotransformation enzymes and some immune parameters but did not generate any DNA damage. The proteomics study showed that several pathways were disturbed in contaminated fish including energy metabolism, methionine metabolism and detoxification system. Studies on natural populations confirmed the alteration of the immune system and of the endocrine system (including the egg-yolk production) in fish from the Seine despite any significant impact on the growth parameters and the condition index but with a strong impact on the genetic diversity (heterozygosity). Isotopic measurements highlighted the importance of environmental factors for the fish physiology and thus the corresponding biomarkers. In particular, the inter-estuarine variability of temperature, hypoxic conditions and development strategies (where flounder can live alternatively in marine or fresh water) can greatly influence the responses measured. These isotopic measurements thus provide a unique information that gives a different perspective and allow to understand how the various situations might have consequences on the flounder populations.

Thus, this integrative study clearly showed that physiological mechanisms are affected in polluted environments and in particular in the Seine. Metabolism, immune system and endocrine system are specific targets that can be studied using sensitive and informative biomarkers. Interestingly, this did not result in clear effects on more integrative measures such as growth, but seem to be associated with changes in the genetic heritage, in particular an increase in the diversity that could promote resistance to survivors.

Implementation of biomarkers for the management of aquatic systems

In the third part of the DIESE program, the research aimed at developing and testing a biomaker approach to support the environmental management. Two lanes of specific studies have been followed. The first was to implement over all or part of the study area, robust biomarkers to establish a mapping that may highlight water bodies at risk and help to identify the sources of biological effects. The second task was devoted to development of methodologies for the use of biomarkers and their integration into a simple and clear index.

Considering the development of robust tools, we conducted an analysis of the relevance of the use of gene expression quantification using molecular biology tools and especially the quantitative PCR which is more and more used and widespread among research groups. This rigorous study has shown that the quantification could quickly lead to erroneous interpretations of the results if certain conditions were not met. In particular the choice of a sufficient number of reference genes is important. Our recommendation would be to ensure that an appropriate preliminary assessment of the genetic stability of the reference genes has been performed before each qPCR quantification and that a sufficient number (e.g. 6) of these control genes has been used to ensure the reliability of the results (Ciocan et al. 2011).

The integration of biomarkers into a broader strategy to assess the quality of the environment strategy is a major challenge. This strategy has not yet any defined role in the European regulations although it may provide relevant information on key parameters such as the metabolism, the reproduction or the immune status of the living organisms, these very parameters that are at risk even considering the human population. Biomarkers may provide a biological meaning to the presence of environmental contaminants and integrate a sum of factors to help building a coherent picture of the stressors and effects.

An integrative study was conducted on juvenile flounder using both laboratory-based and field studies. A suite of molecular, physiological and biometric biomarkers have been implement. This study highlighted the importance of deploying markers related to different levels of the biological organisation as they provide complementary information even if they sometimes give a slightly ambivalent image that is characteristic of biological reactions. It showed that some biochemical biomarkers, in particular those related to the metabolism, may give ambivalent responses and show

important variability that requires the use of a series of markers. The physiological biomarkers appeared more predictive of adverse effects of chemical contaminants. The use of biomarkers with high ecological relevance are relevant for assessing the effects of chemical contaminants on the health of organisms and even more populations. The use of growth, the Fulton condition index, RNA/DNA ratio and lipid index (TAG/ST) have been successfully used to assess the effects of contaminants chemicals on juvenile fish in both field studies and laboratory experiments. They indirectly inform on energy costs induced by chemical contaminants (Adams et al., 1990) and can reflect the probability of survival of the organism. These biological indices have the advantage of being relatively easy to measure and provide relevant information. In addition, this study demonstrated the potential of the use of juvenile fish in ecotoxicological studies in relation to their physiological performance which are higher than those of the older animals.

The integration of multiple biomarkers into a simple index that may provide a simple and clear information derived from multiple biological responses was a particular objective of the DIESE program. The biomarker index (IBI) was developed based on the existing IBR (Integrated Biological Responses) developed by Beliaeff and Burgeot (2002). The work showed that it is necessary to fully characterize the biological responses in order to know its natural variations and thus to identify changes indicative of disturbances. The developed tool integrated positive and negative changes to draw a graph that clearly identify the biological effects. The tool is scalable and can evolve to integrate new biomarkers and thus refine the generated information.

Some biomarkers provide very clear and relevant information which can be related to physiological consequences or even ecological consequences. Some of these biological responses have been implemented during the DIESE program to provide information on the state of the environment and organisms in the Manche region. Partial or whole-region maps of the Channel area were produced to report the presence of mutagenic and anti-androgenic compounds in the sediments of French and British coasts but also to show the occurrence of intersex roach and *S. plana* as well as imposex in *N. lapillus*. The generated maps may remain to be completed to some extent and some more work will be necessary to confront the information in order to draw relevant conclusions for the management of the environment, a goal that the DIESE program has pursued and contributed to by providing some original and necessary information.

V. REFERENCES

Adams S.M., Shugart, L.R., Southworth, G.R., Hinton, D.E., 1990. Application of bioindicators in assessing the health of fish populations experiencing contaminant stress. In: McCarthy, J.F., Shugart, L.R. (Eds.), Biomarkers of Environmental Contamination. Lewis Publishers, Boca Raton, pp. 333–353 (Chapter 19).

Adams SB, Schmetterling DA. 2007. Freshwater sculpins: Phylogeneties to ecology. Transactions of the American Fisheries Society 136 (6):1736-1741.

Akcha F., Vincent F., Hubert Pfhol-Leszkowicz A. 2003. «Potential value of the comet assay and DNA adduct measurement in dab (*Limanda limanda*) for assessment of in situ exposure to genotoxic compounds». *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, vol. 534, no 1–2, p. 21-32.

Allen Y, Scott AP, Matthiessen P, Haworth S, Thain JE, Feist S. 1999. Survey of estrogenic activity in United Kingdom estuarine and coastal waters and its effects on gonadal development of the flounder *Platichthys flesus*. Environmental Toxicology and Chemistry 18 (8):1791-1800.

Alquezar R., Markich, S.J., Booth, D.J. 2006. Effects of metals on condition and reproductive output of the smooth toadfish in Sydney estuaries, south−eastern Australia. Environmental Pollution 142, 116–122.

Al-Yakoob S.M., Gundersen, D., Curtis, L. 1996. Effects of the Water-Soluble Fraction of Partially Combusted Crude Oil from Kuwait's Oil Fires (from Desert Storm) on Survival and Growth of the Marine Fish *Menidia beryllina*. Ecotoxicology and Environmental Safety 35, 142–149.

Amara R., Meziane T., Gilliers C., Hermel G., Laffargue P. 2007. Growth and condition indices in juvenile sole *Solea solea* measured to assess the quality of essential fish habitat. Marine Ecology Progress Series 351:201-208. doi:10.3354/meps07154.

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

Arukwe A., Goksoyr A. 1998. Xenobiotics, xenoestrogens and reproduction disturbances in fish. Sarsia 83 (3):225-241.

Arukwe A., Goksøyr A. 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. comparative hepatology 2:1-21.

Arukwe A., Knudsen F.R., Goksøyr A. 1997. Fish zona radiata (eggshell) protein: a sensitive biomarker for environmental estrogens. Environmental Health Perspectives 105(4):418-418.

Babo S., Vasseur P. 1992. In vitro effects of thiram on liver antioxidant enzyme activities of rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 22, 61–68.

Batty J, Lim R. 1999. Morphological and Reproductive Characteristics of Male Mosquitofish (*Gambusia affinis holbrooki*) Inhabiting Sewage-Contaminated Waters in New South Wales, Australia. Archives of Environmental Contamination and Toxicology 36 (3):301-307.

Baumard P., Budzinski H., Garrigues P. 1997. Analytical procedure for the analysis of PAHs in biological tissues: application to mussels. Fresenius Z. Analytical Chemistry 359, 502–509.

Beck M.W., Heck K., Able K., Childers D., Egglestone D., Gillanders B., Halpern B., Hays C., Hoshino K., Minello T., Orth R., Sheridan P., Weintein M. 2001. The identification, conservation and management of estuarine and marine nurseries for fish and invertebrates. Bioscience 51, 633–641.

Beeby A. 2001. What do sentinels stand for? Environmental Pollution 112 (2):285-298.

Bessho H, Iwakami S, Hiramatsu N, Hara A, Hashimoto S. 2000. Development of a sensitive luminometric immunoassay for determining baseline seasonal changes in serum vitellogenin levels in male flounder (*Pleuronectes yokohamae*). International Journal of Environmental Analytical Chemistry 76 (3):155-166.

Beyers D., Rice J., Clements W., Henry C. 1999. «Estimating physiological cost of chemical exposure: integrating energetics and stress to quantify toxic effects in fish». *Can. J. Fish. Aquat. Sci*, vol. 56, p. 814-822.

Bilbao E., Raingeard D., Diaz de Cerio O., Ortiz-Zarragoitia M., Ruiz P., Izagirre U., Orbea A., Marigomez I., Cajaraville M.P., Cancio I.. 2010. Effects of exposure to Prestige-like heavy fuel oil and to perfluorooctane sulfonate on conventional biomarkers and target gene transcription in the thicklip grey mullet *Chelon labrosus*. Aquatic Toxicology 98, 282–296.

Billen G., Garnier J., Ficht A., Cun C. 2001. Modeling the response of water quality in the Seine River estuary to human activity in its watershed over the last 50 years. Estuaries, 24(6):977-993.

Binder-Foucard F, Belot A, Delafosse P, Remontet L, Woronoff A-S, Bossard N. 2013. Estimation nationale de l'incidence et de la mortalité par cancer en France entre 1980 et 2012. Partie 1 - Tumeurs solides. Saint-Maurice (Fra) : Institut de veille sanitaire. 122 p.

Birchenough, A.C., Evans, S.M., Moss, C. & Welch, R. 2002 Re-colonisation and recovery of populations of dogwhelks Nucella lapillus (L.) on shores formerly subject to severe TBT contamination.Marine Pollution Bulletin. 44 (7); 652-659.

Bjerregaard LB, Korsgaard B, Bjerregaard P. 2006. Intersex in wild roach (*Rutilus rutilus*) from Danish sewage effluent-receiving streams. Ecotox Environ Safe 64 (3):321-328.

Bjorkblom C, Hogfors E, Salste L, Bergelin E, Olsson PE, Katsiadaki I, Wiklund T. 2009. Estrogenic and Androgenic Effects of Municipal Wastewater Effluent on Reproductive Endpoint Biomarkers in Three-Spined Stickleback (*Gasterosteus aculeatus*). Environmental Toxicology and Chemistry 28 (5):1063-1071.

Blaise C., Gagné F., Salazar M., Salazar S., Trottier S., Hansen P.D. 2003. Experimentally-induced feminisation of freshwater mussels after long-term exposure to a municipal effluent. Fresenius Environ. Bull. 12, 8.

Blanchard, M., Teil, M.J., Carru, A.M., Chesterikoff, A., Chevreuil, M. 1997. Organochlorine distribution and mono-orthosubstituted PCB pattern in the roach (*Rutilus rutilus*) from the river Seine. Water Res, 31:1455-1461.

Bligh, E.G., Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, 37, 911–917.

Bols, N.C., J.L. Brubacher, R.C. Ganassin et L.E.J. Lee. 2001. «Ecotoxicology and innate immunity in fish». *Developmental & Comparative Immunology*, vol. 25, no 8-9, p. 853-873.

Bon E, Barbe U, Rodriguez JN, Cuisset B, Pelissero C, Sumpter JP, LeMenn F. 1997. Plasma vitellogenin levels during the annual reproductive cycle of the female rainbow trout (Oncorhynchus mykiss): Establishment and validation of an ELISA. ComparativeBiochemistry and Physiology B-Biochemistry & Molecular Biology 117 (1):75-84.

Borg B. 1993. Effectiveness of several androgens in stimulating kidney hypertrophy, a secondary sexual character, in castrated male three-spined sticklebacks, *Gasterosteus aculeatus*. Can J Zool: 2327-2329.

Bradford, M.M., 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilising the principle of protein–dye binding. Analytical Biochemistry 72, 248–254.

Bray, S., Herbert, R.J.H. 1998. A reassessment of populations of the dog-whelk (Nucella lapillus) on the Isle of Wight following legislation restricting the use of TBT antifouling paints. Proceedings of the Isle of Wight Natural History and Archaeological Society. 14; 23-40.

Brion F, Nilsen BM, Eidem JK, Goksoyr A, Porcher JM. 2002. Development and validation of an enzyme-linked immunosorbent assay to measure vitellogenin in the zebrafish (*Danio rerio*). Environmental Toxicology and Chemistry 21 (8):1699-1708.

Brion F, Rogerieux F, Noury P, Migeon B, Flammarion P, Thybaud E, Porcher JM. 2000. Two-step purification method of vitellogenin from three teleost fish species: rainbow trout (*Oncorhynchus mykiss*), gudgeon (*Gobio gobio*) and chub (*Leuciscus cephalus*). Journal of Chromatography B 737 (1-2):3-12.

Bryan, G.W. Gibbs. P.E., Burt, G.R. and Hummerstone, L.G. 1986. The decline of the gastropod Nucella lapillus around South-West England: evidence for the effects of tributllyltin from anti-fouling paints. Journal of the Marine Biological Association of the United Kingdom 66:611-640

Bucher F, Hofer R, Krumschnabel G, Doblander C. 1993. Disturbances in the Prooxidant - Antioxidant Balance in the Liver of Bullhead (*Cottus-gobio* L) Exposed to Treated Paper-Mill Effluents. Chemosphere 27 (8):1329-1338.

Bucher F, Hofer R, Salvenmoser W. 1992. Effects of Treated Paper-Mill Effluents on Hepatic Morphology in Male Bullhead (*Cottus-gobio* L). Archives of Environmental Contamination and Toxicology 23 (4):410-419.

Bucher F, Hofer R. 1993. Histological and Enzyme Histochemical-Changes in the Kidney of Male Bullhead (*Cottus Gobio*) During the Spawning Period. Journal of Fish Biology 42 (3):403-409.

Buckley L., Caldarone E., Ong T.L. 1999. RNA–DNA ratio and other nucleic acid−based indicators for growth and condition of marine fishes. Hydrobiologia 401, 265–277.

Budzinski H., Jones I., Bellocq J., Piérard C., Garrigues P. 1998. Evaluation of the sediment contamination by PAHs in the Gironde estuary. *Mar. Chem*., 58: 85-97.

Budzinski H., Mazeas O., Tronczynski J., Desaunay Y., Bocquene Claireaux G. 2004. Link between exposure of fish (*Solea solea*) to PAHs and metabolites: application to the ‘‘Erika’’ oil spill. Aquatic Living Resources 17, 329–334.

Bugel S.M., White L.A., Cooper K.R. 2010. Impaired reproductive health of killifish (*Fundulus heteroclitus*) inhabiting Newark Bay NJ, a chronically contaminated estuary. Aquat Toxicol, 96 (3):182–193.

Cachot J., Geffard O., Augagneur S., Lacroix S., Le Menach K., Peluhet L., et al., 2006. Evidence of genotoxicity related to high PAH content of sediments in the upper part of the Seine estuary (Normandy, France). *Aquatic Toxicol*. 79 : 257–267.

Caporal-Gautier J, Nivet JM AP, Guilloteau M, Histe M, M. L. 1992b. Guide de validation analytique. Rapport d’une commission SFSTP. I. Me´thodologie. STP Pharma Pratiques 2:205-226.

Caporal-Gautier J, Nivet JM, Algranti P, Guilloteau M, Histe M, M. L. 1992a. Guide de validation analytique. Rapport d'une commission SFSTP. II. Exemples d'application. . STP Pharma Pratiques 2:227-239.

Castro B.B., Sobral O. Guilhermino L., Ribeiro R. 2004. An In Situ Bioassay Integrating Individual and Biochemical Responses Using Small Fish Species. Ecotoxicology 13, 667–681.

CCME. 1999. Canadian sediment quality guidelines for the protection of aquatic life: Summary tables. In: Canadian environmental quality guidelines, 1999, Canadian Council of Ministers for the Environment, Winnipeg.

Chesman, B. S.; Langston, W. J., Intersex in the clam Scrobicularia plana: a sign of endocrine disruption in estuaries? *Biol*

Christiansen LB, Pedersen KL, Pedersen SN, Korsgaard B, Bjerregaard P. 2000. In vivo comparison of xenoestrogens using rainbow trout vitellogenin induction as a screening system. Environmental Toxicology and Chemistry 19 (7):1867-1874.

Claireaux, G., Désaunay, Y., Akca, F., Aupérin, B., Bocquené, G., Budzinski, H., Cravedi, J.P., Davoodi, F., Galois, R., Gilliers, C., Goanvec, C., Guérault, D., Imbert, N., Mazéas, O., Nonotte, G., Nonotte, L., Prunet, P., Sébert P., Vettier, A. 2004. Influence of oil exposure on the physiology and ecology of the common sole *Solea solea*: experimental and field approaches. Aquatic Living Resources 17, 335–351.

Clemmesen C.M. 1988. A RNA and DNA fluorescence technique to evaluate the nutritional condition of individual marine fish larvae. Meeresforschung, Reports on Marine Research 32, 134–143.

Colborn T, Saal FSV, Soto AM. 1993. Developmental Effects of Endocrine-Disrupting Chemicals in Wildlife and Humans. Environmental Health Perspectives 101 (5):378- 384.

Collier T.K., Krone C.A., Krahn M.G., Stain J.E., Chan S.L., Varanasi U. 1996. Petroleum exposure and associated biochemical effects in subtidal fish after the Exxon valdez oil spill. American Fisheries Society Symposium 18, 671–683.

Colson, I. and Hughes, R.N. 2004. Rapid recovery of genetic diversity of dogwhelk (Nucella lapillus L.) populations after local extinction and recolonisation contradicts predictions from life history characteristics. Molecular Ecology. 13; 2223-2233.

Cravatt B. F., Simon G. M., Yates J. R. 2007. The biological impact of mass-spectrometry-based proteomics. Nature 450(7172):991-1000.

De Wit, M., D. Keil, N. Remmerie, K. Van der Ven, E. J. Van den Brandhof, D. Knapen, E. Witters & W. De Coen, 2008. Molecular targets of TBBPA in zebrafish analysed through integration of genomic and proteomic approaches. Chemosphere 74(1):96–105.

DeFranco, A.L., M. Robertson et R.M. Locksley. 2009. *Immunité : la réponse immunitaire dans les maladies infectieuses et inflammatoires*. Bruxelles.

Defur, P., Crane, M., Ingersoll, C. & Tattersfield, L. 1999. Endocrine Disruption in Invertebrates: Endocrinology, Testing and Assessment. Pensacola, FL.

Delgado, M. & Camacho, A. P. 2002; Hermaphroditism in Ruditapes decussatus (L.)(Bivalvia) from the Galician coast (Spain). Scientia Marina 66, 183-185.

Déniel, C. 1981. «Les Poissons plats (Téléostéens, Pleuronectiformes) en baie de Douarnenez: reproduction, croissance et migration des *Bothidae*, *Scophthalmidae, Pleuronectidae* et *Soleidae*». Université de Bretagne Occidentale.

Depledge, M.H., Aagaard, A., Gyorkos, P., 1995. Assessment of trace metal toxicity using molecular, physiological and behavioural biomarkers. Marine Pollution Bulletin 31 (1-3), 19–27.

Dorts J, Kestemont P, Dieu M, Raes M, Silvestre F. 2011a. Proteomic Response to Sublethal Cadmium Exposure in a Sentinel Fish Species, *Cottus gobio*. Journal of Proteome Research 10 (2):470-478.

Dorts J, Kestemont P, Marchand P-A, D'Hollander W, Thezenas M-L, Raes M, Silvestre F. 2011b. Ecotoxicoproteomics in gills of the sentinel fish species, *Cottus gobio*, exposed to perfluorooctane sulfonate (PFOS). Aquatic Toxicology 103 (1-2):1-8.

Driedger K., Weber L.P., Rickwood C.J., Dube M.G., Janz D.M. 2010. Growth and energy storage in juvenile fathead minnows exposed to metal mine waste water in simulated winter and summer conditions. Ecotoxicology and Environmental Safety 73, 727–734.

Dufour E, Gerdeaux D, Wurster CM. 2007. Whitefish (*Coregonus lavaretus)* respiration rate governs intra-otolith variation of $\delta^{13}C$ values in Lake Annecy. Canadian Journal of Fisheries Aquatic Sciences, **64**, 1736-1746.

Dunier M. 1996. «effets des pesticides et des metaux lourds sur le système immunitaire de la carpe *Cyprinus carpio*». *Ichtyophysiol*., vol. Acta 14, p. 9-22.

Dunier M. et A.K. Siwicki. 1993. Effects of pesticides and other organic pollutants in the aquatic environment on immunity of fish - a review. *Fish & Shellfish Immunology*, vol. 3, no 6, Nov, p. 423-438.

Evans, S.M., Evans, P.M. & Leksono, T. 1996. Widespread recovery of dogwhelks, Nucella lapillus (L.), from tributyltin contamination in the North Sea and Clyde Sea Mar.Poll.Bull., 32, 263-369.

Evans, S.M., Hawkins, S.T., Porter, J. & Samosir, A.M. 1994. Recovery of dogwhelk populations on the Isle of Cumbrae, Scotland, following legislation limiting the use of TBT as an antifoulant. Mar.Poll.Bull. 28(1): 15-17.

Evans, S.M., Kerrigan, E. & Palmer, N. 2000. Causes of imposex in the dogwhelk Nucella lapillus (L.) and its use as a biological indicator of tributyltin contamination. Mar.Poll.Bull. 40:212-219.

Evans, S.M., Leksono, T. & McKinnell, P.D. 1995. Tributyltin pollution: a diminishing problem following legislation limiting the use of TBT-based anti-fouling paints. Mar.Poll.Bull. 30(1): 14-21.

Evrard E. 2009. «Réponses d'un poisson estuarien le flet (*Platichthys flesus*) au stress pseticides en milieu expérimental et au multi-stress in situ». Université de Bretagne Occidentale, 189 p.

Evrard E., A. Devaux, S. Bony, J. Cachot, G. Charrier, L. Quiniou et J. Laroche. 2012. Responses of juvenile European flounder (*Platichthys flesus*) to multistress in the Vilaine estuary, during a 6-month survey. *Environmental Science and Pollution Research*, p. 1-14.

Evrard E., A. Devaux, S. Bony, T. Burgeot, R. Riso, H. Budzinski, M. Du, L. Quiniou et J. Laroche. 2010a. Responses of the European flounder *Platichthys flesus* to the chemical stress in estuaries: load of contaminants, gene expression, cellular impact and growth rate. *Biomarkers*, vol. 15, no 2, p. 111-127.

Evrard E., Marchand J., Theron M., Pichavant-Rafini K., Durand G., Quiniou L., Laroche J. 2010b. Impacts of mixtures of herbicides on molecular and physiological responses of the European flounder *Platichthys flesus*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, vol. 152, no 3, p. 321-331.

Faller P, Kobler B, Peter A, Sumpter JP, Burkhardt-Holm P .2003. Stress status of gudgeon (*Gobio gobio*) from rivers in Switzerland with and without input of sewage treatment plant effluent. Environmental Toxicology and Chemistry 22 (9):2063-2072.

Flammarion P, Brion F, Babut M, Garric J, Migeon B, Noury P, Thybaud E, Tyler CR, Palazzi X, Tyler CR. 2000. Induction of fish vitellogenin and alterations in testicular structure: Preliminary results of estrogenic effects in chub (*Leuciscus cephalus*). Ecotoxicology 9 (1-2):127-135.

Flammarion P., Garric J. 1997. Cyprinids EROD activities in low contaminated rivers: a relevant statistical approach to estimate reference levels for EROD biomarker. Chemosphere 35, 2375-2388.

Fonseca V., Serafim, A., Compagny, R., Bebianno, M.J., Cabral, H. 2009. Effect of copper exposure on growth, condition indices and biomarker response in juvenile sole *Solea senegalensis*. Scientia Marina 73, 51–58.

França S., M.J. Costa & H.N. Cabral. 2011. Inter- and intra-estuarine fish assemblage variability patterns along the Portuguese coast. Estuarine Coastal and Shelf Science 91: 262-271.

Freyhof J, Kottelat M, Nolte A. 2005. Taxonomic diversity of European Cottus with description of eight new species (Teleostei: Cottidae). Ichthyological Exploration of Freshwaters 16 (2):107-172.

Fukada H, Haga A, Fujita T, Hiramatsu N, Sullivan CV, Hara A. 2001. Development and validation of chemiluminescent immunoassay for vitellogenin in five salmonid species. Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology 130 (1):163-170.

Fulton T.W., 1904. The rate of growth of fishes. Fisheries Board of Scotland, Annual Report 22 part 3, 141–241.

García-Mayor R.V., Vidal A.L., Docet Caamaño M.F., Giménez A.L. 2012. Endocrine disruptors and obesity: Obesogens. *Endocrinol Nutr*. 59(4): 261-267.

Gauthier-Clerc S., Pellerin J., Amiard J.C. 2006. Estradiol-17β and testosterone concentrations in male and female Mya arenaria (Mollusca bivalvia) during the reproductive cycle. Gen. Comp. Endocrinol. 145, 133–139.

Geraudie P, Gerbron M, Hill E, Minier C. 2010a. Roach (*Rutilus rutilus*) reproductive cycle: a study of biochemical and histological parameters in a low contaminated site. Fish Physiology and Biochemistry 36 (3):767-777.

Geraudie P., Boulange-Lecomte C., Gerbron, M., Hinfray, N., Brion, F., Minier, C. 2010b. Endocrine effects of the tapeworm Ligula intestinalis in its teleost host, the roach (Rutilus rutilus). Parasitology, 137(4):697-704.

Geraudie P., Hinfray N., Gerbron M., Porcher J.M., Brion F., Minier C. 2011. Brain cytochrome P450 aromatase activity in roach (*Rutilus rutilus*): seasonal variations and impact of environmental contaminants. Aquat Toxicol, 105(3-4):378-84.

Gercken J, Sordyl H. 2002. Intersex in feral marine and freshwater fish from northeastern Germany. Marine Environmental Research 54 (35):651-655. Gosselin MP, Petts GE, Maddock IP (2010) Mesohabitat use by bullhead (*Cottus gobio*). Hydrobiologia 652 (1):299-310.

Gibbs, P.E., Bryan, G.W., Pascoe, P.L. and Burt, G.R. 1987. The use of the dog-whelk, Nucella lapillus, as an indicator of tributyltin (TBT) contamination. Journal of the Marine Biological Association of the United Kingdom, 67, 507-523.

Gibbs, P.E., Green, J.C. and Pascoe, P.L. 1999. A massive summer kill of the dog-whelk, *Nucella lapillus*, on the north Cornwall coast in the summer of 1995: freak or forerunner?. Journal of the Marine Biological Association of the United Kingdom, 79(1), 103-109

Gibson M.R. 1994. Population dynamics of winter flounder in Mt. Hope Bay in relations to operations at the Brayton Point Electric Plant. RI Division Fish and Wildlife. Report to the Brayton Point Technical Advisory Committee, May 1992. Revised December 1993.

Gibson R.N. 2005. Flatfishes.Biology and Exploitation.Blackwell Publishing, Oxford pp. 1–391.

Gravato C., Santos, M.A. 2003. *Dicentrarchus labrax* biotransformation and genotoxicity responses after exposure to a secondary treated industrial/urban effluent. Ecotoxicology and Environmental Safety 55, 300–306.

Gray MA, Niimi AJ, Metcalfe CD. 1999. Factors affecting the development of testis-ova in medaka, Oryzias latipes, exposed to octylphenol. Environmental Toxicology and Chemistry 18 (8):1835-1842.

Grosbois C., Meybeck M., Horowitz, A., Ficht, A., 2006. Spatio-temporal distribution of Cd, Cu, Hg, Pb and Zn (1984-2000) in the Seine river particulate matter. Sci. Tot. Env, 356(1-3):22-37.

Guérit I., Bocquené G., James A., Thybaud E., Minier C. 2008. Environmental risk assessment: a critical approach of the European TGD in an in situ application. Ecotoxicology and Environmental Safety. 71: 291-300.

Habig W.H., Pabst M.J., Jakoby W.B. 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. Journal of Biology and Chemistry 249, 7130–7139.

Hagger J.A., M.B. Jones, D. Lowe, D.R.P. Leonard, R. Owen et T.S. Galloway. 2008. Application of biomarkers for improving risk assessments of chemicals under the Water Framework Directive: A case study. *Marine Pollution Bulletin*, vol. 56, no 6, p. 1111-1118.

Head J.A.,. Dolinoy D C., Basu N. 2012. Epigenetics for ecotoxicologists. Environmental toxicology and chemistry / SETAC 31(2):221-7 doi:10.1002/etc.1707.

Hecker M, Tyler CR, Hoffmann Me, Maddix S, Karbe L. 2002. Plasma Biomarkers in Fish Provide Evidence for Endocrine Modulation in the Elbe River, Germany. Environmental Science & Technology 36 (11):2311-2321.

Hentschel H (1982) Histochemical-Observations on Glycoproteins in the Urinary Apparatus of the Bullhead *Cottus gobio* L and the Bull Rout *Myoxocephalus scorpius* (L). Zeitschrift Fur Mikroskopisch-Anatomische Forschung 96 (1):171-179.

Hill, E. M.; Evans, K. L.; Horwood, J.; Rostkowski, P.; Oladapo, F. O.; Gibson, R.; Shears, J. A.; Tyler, C. R., Profiles and Some Initial Identifications of (Anti)Androgenic Compounds in Fish Exposed to Wastewater Treatment Works Effluents. *Environ*. *Sci*. *Technol*. 2010, *44*, (3), 1137-1143.

Hinfray N, Palluel O, Piccini B, Sanchez W, Ait-Aissa S, Noury P, Gomez E, Geraudie P, Minier C, Brion F, Porcher JM. 2010. Endocrine disruption in wild populations of chub (*Leuciscus cephalus*) in contaminated French streams. Science of the Total Environment 408 (9):2146-2154.

Hinfray N., Porcher J.-M., Brion F. 2006. Inhibition of rainbow trout (*Oncorhynchus mykiss*) P450 aromatase activities in brain and ovarian microsomes by various environmental substances. Comp. Biochem. Physiol., C. 144:252–262.

Holland M.C.H. et J.D. Lambris. 2002. The complement system in teleosts. *Fish & Shellfish Immunology*, vol. 12, no 5, p. 399-420.

Horowitz A. J.; Meybeck, M.; Idlafkih, Z.; Biger, E., 1999. Variations in trace element geochemistry in the Seine River Basin based on floodplain deposits and bed sediments. Hydrol Process, 13:1329-1340.

Huet M., Y. M. Paulet & J. Clavier. 2004. Imposex in Nucella lapillus: a ten year survey in NW Brittany. Mar Ecol Prog Ser. 270: 153–161.

Hughes, R. 1971. Reproduction of Scrobicularia plana Da Costa (Pelecypoda: Semelidae) in North Wales. Veliger. 14, 77-81.

Hurter E, Pool EJ, Van Wyk JH. 2002. Validation of an *ex vivo Xenopus* liver slice bioassay for environmental estrogens and estrogen mimics. Ecotoxicol Environ Saf 53(1):178-187.

Janer, G.; Bachmann, J.; Oehlmann, J.; Schulte-Oehlmann, U.; Porte, C., The effect of organotin compounds on gender specific androstenedione metabolism in the freshwater ramshorn snail Marisa cornuarietis. *J*. *Steroid Biochem*. *Mol*. *Biol*. 2006, *99*, (2-3), 147-156.

Jobling S, Beresford N, Nolan M, Rodgers-Gray T, Brighty GC, Sumpter JP, Tyler CR. 2002. Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluents. Biology of Reproduction 66 (2):272-281.

Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP. 1998. Widespread sexual disruption in wild fish. Environmental Science & Technology 32 (17):2498-2506. Jobling S, Tyler CR (2003) Endocrine disruption in wild freshwater fish. Pure and Applied Chemistry 75 (11-12):2219-2234.

Jobling S., Tyler C. R. 2003. Endocrine disruption in wild freshwater fish. Pure Appl Chem, 75(11-12):2219-2234.

Jobling, S.; Burn, R. W.; Thorpe, K.; Williams, R.; Tyler, C., Statistical Modeling Suggests that Antiandrogens in Effluents from Wastewater Treatment Works Contribute to Widespread Sexual Disruption in Fish Living in English Rivers. *Environ*. *Health Perspect*. 2009, *117*, (5), 797-802.

Jobling, S.; Williams, R.; Johnson, A.; Taylor, A.; Gross-Sorokin, M.; Nolan, M.; Tyler, C. R.; van Aerle, R.; Santos, E.; Brighty, G., Predicted exposures to steroid estrogens in UK rivers correlate with widespread sexual disruption in wild fish populations. *Environ. Health Perspect.* 2006, *114*, 32-39.

Johnson L.L., Lomax D.P., Myers M.S., Olson O.P., Sol S.Y., O'Neill S.M., West J., Collier T.K. 2008. Xenoestrogen exposure and effects in English sole (*Parophrys vetulus*) from Puget Sound, WA. Aquat Toxicol. 88(1):29-38.

Johnson LL, Casillas E, Collier TK, McCain BB, Varanasi U. 1988. Contaminant effects on ovarian development in English Sole (*Parophrys-vetulus*) from Puget Sound, Washington. Canadian Journal of Fisheries and Aquatic Sciences 45 (12):2133-2146.

Jolly S, Bado-Nilles A, Lamand F, Turies C, Chadili E, Porcher J-M, Betoulle S, Sanchez W. 2012. Multi-biomarker approach in wild European bullhead, *Cottus sp*. exposed to agricultural and urban environmental pressures: Practical recommendations for experimental design. Chemosphere 87 (7):675-683.

Kalish J.M. 1991. ^{13}C and ^{18}O isotopic disequilibria in fish otoliths: metabolic and kinetic effects. *Marine Ecology Progress Series,* vol. 75, p. 191-203.

Karels A, Soimasuo M, Oikari A. 1999. Effects of pulp and paper mill effluents on reproduction, bile conjugates and liver MFO (mixed function oxygenase) activity in fish at Southern Lake Saimaa, Finland. Water Science and Technology 40 (11-12):109-114.

Karels AA, Manning S, Brouwer TH, Brouwer M. 2003. Reproductive effects of estrogenic and antiestrogenic chemicals on sheepshead minnows (*Cyprinodon variegatus*). Environmental Toxicology and Chemistry 22 (4):855-865.

Katsiadaki I, Morris S, Squires C, Hurst MR, James JD, Scott AP. 2006. Use of the threespined stickleback (*Gasterosteus aculeatus*) as a sensitive in vivo test for detection of environmental antiandrogens. Environmental Health Perspectives 114:115-121.

Katsiadaki I, Scott AP, Hurst MR, Matthiessen P, Mayer I. 2002. Detection of environmental androgens: A novel method based on enzyme-linked immunosorbent assay of spiggin, the stickleback (*Gasterosteus aculeatus*) glue protein. Environmental Toxicology and Chemistry 21 (9):1946-1954.

Katsiadaki I, Scott AP, Mayer I. 2002. The potential of the three-spined stickleback (*Gasterosteus aculeatus* L.) as a combined biomarker for oestrogens and androgens in European waters. Marine Environmental Research 54 (3-5):725-728.

Ketata I. Denier X., Chaffai A. & Minier C. 2008. Endocrine-related reproductive effects in molluscs. *Comp. Biochem. Physiol*. 147: 261-270.

Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, Flick RW. 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc Natl Acad Sci U S A 104 (21):8897-8901.

Kime DE, Nash JP, Scott AP. 1999. Vitellogenesis as a biomarker of reproductive disruption by xenobiotics. Aquaculture 177(1-4):345-352.

Kime, D. E., 1998. Endocrine Disruption in Fish. Kluwer Academic, Boston. 396.

Kirby MF, Allen YT, Dyer RA, Feist SW, Katsiadaki I, Matthiessen P, Scott AP, Smith A, Stentiford GD, Thain JE, Thomas KV, Tolhurst L, Waldock MJ. 2004. Surveys of plasma vitellogenin and intersex in male flounder (*Platichthys flesus*) as measures of endocrine disruption by estrogenic contamination in United Kingdom estuaries: Temporal trends, 1996 to 2001. Environmental Toxicology and Chemistry 23 (3):748-758.

Kling P., Norman A., Andersson P. L., Norrgren L., Förlin L. 2008. Gender-specific proteomic responses in zebrafish liver following exposure to a selected mixture of brominated flame retardants. Ecotoxicology and Environmental Safety 71:319–327.

Labadie, P.; Cundy, A. B.; Stone, K.; Andrews, M.; Valbonesi, S.; Hill, E. M., Evidence for the migration of steroidal estrogens through river bed sediments. 2007, *41*, (12), 4299-4304.

Labadie, P.; Hill, E. M., Analysis of estrogens in river sediments by liquid chromatography-electrospray ionisation mass spectrometry - Comparison of tandem mass spectrometry and time-of-flight mass spectrometry. 2007, *1141*, (2), 174-181.

Lacroix A., Fournier M., Lebeuf M., Nagler J.J., Cyr D.G. 2001. «Phagocytic response of macrophages from the pronephros of American plaice (*Hipoglossoides platessoides*) exposed to contaminated sediments from Baie des Anglais, Quebec». *Chemosphere*, vol. 45, no 4-5, Nov, p. 599-607. En ligne: <Go to ISI>://000171451900022.

Lafite R., Romana A. 2001. A man-altered macrotidal estuary: the Seine estuary (France): introduction to the special issue Estuaries, 24:939.

Langston, W. J., Pope, N. D., Jonas, P. J. C., Nikitic, C., Field, M. D. R., Dowell, B., Shillabeer, N., Swarbrick, R. H. & Browne. A. R. 2010. Contaminants in fine sediments and their consequences for biota of the Severn Estuary. Marine Pollution Bulletin. Volume 61, issues 1-3, 68-82. doi:10.1016/j.marpolbul.2009.12.014

Langston, W., B. Chesman, G. Burt, S. Hawkins, J. Readman & P. Worsfold, 2003. Characterisation of the South West European Marine Sites : Plymouth Sound and Estuaries cSAC, SPA. Occasional Publications. Mar Biol Assoc Occ Pub 9:202p.

Langston, W.J., and Burt, G.R. 2007. A review of TBT sediment data in the Fal and Helford SAC, Unpublished report for the Environment Agency

Langston, W.J., Bryan, G.W., Burt, G.R. and Pope, N.D. 1994. Effects of sediment metals on estuarine benthic organisms, Project Record 105/2/A, National Rivers Authority, 49pp

Langston, W.J., Burt, G.R. and Pope, N.D. 1999. Bioavailability of metals in sediments of the Dogger Bank (central North Sea): a mesocosm study. Estuarine Coastal and Shelf Science, 48, 519-540.

Langston, W.J., Burt, G.R., & Chesman, B.S. 2007. Feminisation of male clams Scrobicularia plana from estuaries in South West UK and its induction by endocrine-disrupting chemicals. Mar. Ecol. Prog. Ser. 333:173-184.

Langston, W.J., Chesman, B.S., Burt, G.R., Hawkins, S.J., Readman J., and Worsfold P. 2003. Characterisation of the South West European Marine Sites: The Fal and Helford cSAC. Marine Biological Association of the U K., Occasional Publication No. 8. pp 160.

Lapointe D., Couture P. 2010. Accumulation and effects of nickel and thallium in early-life stages of fathead minnows (*Pimephales promelas*). *Ecotoxicology and Environmental Safety*, vol. 73, no 4, p. 572-578.

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

Larsson R, Nygren P. 1989. A rapid fluorometric method for semi-automated determination of cytotoxicity and cellular proliferation of human tumor cell lines in microculture. Anticancer Res 9:1111-1120.

Lavado R, Thibaut R, Raldua D, Martin R, Porte C. 2004. First evidence of endocrine disruption in feral carp from the Ebro River. Toxicology and Applied Pharmacology 196 (2):247-257.

Lee R.F., Steinert S. 2003. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. *Mutation Research-Reviews in Mutation Research*, 544: 43-64.

Lesser M.P., Farrell J.H., Walker C.W. 2001. Oxidative stress DNA damage and p53 expression in the larvae of Atlantic cod (*Gadus morhua*) exposed to ultraviolet (290–400 nm) radiation. Journal of Experimental Biology 204, 157–164.

Lett. 2006, *2*, (3), 420-422.

Liney, K. E.; Hagger, J. A.; Tyler, C. R.; Depledge, M. H.; Galloway, T. S.; Jobling, S., Health effects in fish of long-term exposure to effluents from wastewater treatment works. *Environ*. *Health Perspect*. 2006, *114*, 81-89.

Loizeau V., Abarnou A. 1995. Niveaux de contamination par les PCB dans le réseau trophique du bar et du flet. Rapport scientifique Seine Aval, Thème Edifices Biologiques, 85–120.

Lower WR, Kendall RJ (1990) Biomarkers of Environmental Contamination. Boca Raton, USA: Lewis Publishers.

Lu X.-J., J. Chen, Z.-A. Huang, L. Zhuang, L.-Z. Peng et Y.-H. Shi. 2012. Influence of acute cadmium exposure on the liver proteome of a teleost fish, ayu (*Plecoglossus altivelis*). *Molecular Biology Reports*, 39: 2851-2859.

Lyons BP, Thain JE, Stentiford GD, Hylland K, Davies IM, Vethaak AD. 2010. Using biological effects tools to define good environmental status under the European Union Marine Strategy Framework Directive. Mar Poll Bull, 60: 1647-1651.

Ma, T., Wan, X., Huang, Q., Wang, Z., Liu, J., 2005. Biomarker responses and reproductive toxicity of the effluent from a Chinese large sewage treatment plant in Japanese medaka (*Oryzias latipes*). Chemosphere, 59(2):281-288.

Mackie, G. L. 1984. in Vol 7. Reproduction (eds. Wilbur, K. M., Tompa, A. S., Vedonk, N. H. & Van den Biggelar, J. A. M.) 351-417. Acedemic Press, New York.

Maltret-Geraudie P, Gerbron M, Minier C. 2008. Estrogenic response of wild roach from the Seine River (France). Cybium 32(2):256-257.

Maltret-Geraudie P., Gerbron M., Minier C. 2008. Estrogenic response of wild roach from the Seine River (France). Cybium, 32: 256-257.

Marchand J, Denis F, Laroche J. 2013. Evolutionary Toxicology & Transcriptomic Approaches. *In*: Amiard-Triquet C, Amiard JC, Rainbow PS. Ecological Biomarkers: Indicators of Ecotoxicological Effects. CRC Press. 361-383.

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

Marchand J., A. Tanguy G. Charrier L. Quiniou E. Plee-Gauthier, J. Laroche. 2006. Molecular identification and expression of differentially regulated genes of the european flounder, *Platichthys flesus*, submitted to pesticide exposure. *Marine Biotechnology*, 8, 275-294.

Marshall Adams S, Crumby WD, Greeley Jr MS, Shugart LR, Saylor CF. 1992. Responses of fish populations and communities to pulp mill effluents: A holistic assessment. Ecotox Environ Safe 24 (3):347-360.

Martyniuk, C. J., R. J. Griffitt & N. D. Denslow, 2011b. Omics in aquatic toxicology: not just another microarray. Environmental toxicology and chemistry / SETAC 30(2):263-4 doi:10.1002/etc.428.

Masson G. 1987. Biologie et ecologie d'un poisson plat amphihalin, le flet (*Platichthys flesus* Linne, 1758) dans l'environnement ligerien: distribution, demographie, place au sein des reseaux trophiques. Sciences: Brest: 1987.

Mayon N, Bertrand A, Leroy D, Malbrouck C, Mandiki SNM, Silvestre F, Goffart A, Thome J-P, Kestemont P. 2006. Multiscale approach of fish responses to different types of environmental contaminations: A case study. Science of the Total Environment 367 (2-3):715-731.

Meier S., Morton H. C., Andersson E., Geffen A. J., Taranger G. L., Larsen M., Petersen M., Djurhuus R., Klungsøyr J., Svarda l A. 2011. Low-dose exposure to alkylphenols adversely affects the sexual development of Atlantic cod (*Gadus morhua*): Acceleration of the onset of puberty and delayed seasonal gonad development in mature female cod. Aquat Toxicol, 105(1-2):136-150.

Meziane, T., Tsuchiya, M., 2002. Organic matter in a subtropical mangrove- estuary subjected to wastewater discharge: origin and utilization by two macrozoobenthic species. Journal of Sea Research 47(1), 1–11.

Milinkovitch, T., Ndiaye, A., Sanchez, W., Le Floch, S., Thomas-Guyon, H., 2011. Liver antioxidant and plasma immune responses in juvenile golden grey mullet (*Liza aurata*) exposed to dispersed crude oil. Aquatic Toxicology 101, 155–164.

Minagawa M, Wada E. 1984. Stepwise enrichment of $\delta^{15}N$ along food chains: further evidence and the relation between $\delta^{15}N$ and animal age. *Geochimica Cosmochimica Acta*, 48, 1135-1140

Minier C, Caltot G, Leboulenger F, Hill EM. 2000. An investigation of the incidence of intersex fish in Seine-Maritime and Sussex regions. Analusis 28:801-806.

Minier C, Levy F, Rabel D, Bocquene G, Godefroy D, Burgeot T, Leboulenger F. 2000. Flounder health status in the Seine Bay. A multibiomarker study. Marine Environmental Research 50 (1-5):373-377.

Minier C., Abarnou A., Jaouen-Madoulet A., Le Guellec A.M., Tutundjian R., Bocquené G., Leboulenger F. 2006. A pollution-monitoring pilot study involving contaminant and biomarker measurements in the Seine Estuary, France, using zebra mussels (*Dreissena polymorpha*). Environ Toxicol Chem., 25(1):112-119.

Minier C., Amara R. 2008. From pollution to altered fish physiological performance; the case of flatfish in the Seine estuary. In Amiard-Triquet Environmental assessment of estuarine ecosystems. Taylor and Francis. Boca Raton. 227-240.

Moles A., Norcross B.L. 1997. Effects of oil-laden sediments on growth and health of juvenile flatfishes. Canadian Journal of Fisheries and Aquatic Sciences 55, 605–610.

Monod G., De Mones A., Fostier A. 1993. Inhibition of ovarian microsomal aromatase and follicular oestradiol secretion by imidazole fungicides in rainbow trout. Mar Environ Res, 35:153–157.

Monteoliva L. Albar J. P. 2004. Differential proteomics: an overview of gel and non-gel based approaches. Briefings in Functional Genomics & Proteomics, 3, 220–239.

Moore, J.J., James, B., Minchin, A. & Davies, I.M. 2000. Surveys of dog whelks Nucella lapillus in the vicinity of Sullom Voe, Shetland, August 1999. Report to the Shetland Oil Terminal Environmental Advisory Group (SOTEAG), prepared by CORDAH Ltd and the Fisheries Research Services.

Munkittrick KR, McMaster ME, McCarthy LH, Servos MR, Van Der Kraak GJ. 1998. An overview of recent studies on the potential of pulp-mill effluents to alter reproductive parameters in fish. Journal of Toxicology and Environmental Health-Part B-Critical Reviews 1 (4):347-371.

Munschy C., K. Moisan I. Truquet Tronczynski J. 1996. Caractérisation chimique de la contamination organique dans l'estuaire de la Seine. Rapport Seine-Aval 1996/FIN-3 Agence de l'Eau Seine-Normandie, France.

Nadzialek S, Depiereux S, Mandiki SNM, Kestemont P. 2011. In Vivo Biomarkers of Estrogenicity: Limitation of Interpretation in Wild Environment. Archives of Environmental Contamination and Toxicology 60 (3):471-478.

Navas JM, Segner H. 2006. Vitellogenin synthesis in primary cultures of fish liver cells as endpoint for in vitro screening of the (anti)estrogenic activity of chemical substances. Aquat Toxicol 80(1):1-22.

Nilsen BM, Berg K, Edem JK, Kristiansen SV, Brion F, Porcher JM, Goksoyr A. 2004. Development of quantitative vitellogenin-ELISAs for fish test species used in endocrine disruptor screening. Anal Bioanal Chem 378:621-633.

Noaksson E., Gustavsson, B., Linderoth, M., Zebühr, Y., Broman, D., Balk, L., 2004. Gonad development and plasma steroid profiles by HRGC/HRMS during one reproductive cycle in reference and leachate-exposed female perch (*Perca fluviatilis*). Toxicol Appl Pharmacol, 195(2):247-261.

Noaksson E., Linderoth, M., Bosveld, A.T., Balk, L., 2003a. Altered steroid metabolism in several teleost species exposed to endocrine disrupting substances in refuse dump leachate. Gen Comp Endocrinol, 134(3):273-284.

Noaksson E., Tjärnlund, U., Bosveld, A.T., Balk, L., 2001. Evidence for endocrine disruption in perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*) in a remote Swedish lake in the vicinity of a public refuse dump. Toxicol Appl Pharmacol, 174(2):160-176.

Noaksson, E., Linderoth, M., Bosveld, A.T.C., Norrgren, L., Zebühr, Y., Balk, L., 2003b. Endocrine disruption in brook trout (*Salvelinus fontinalis*) exposed to leachate from a public refuse dump. Sci. Total Environ, 305:87-103.

Norton E.C., MacFarlane R.B., Mohr M.S. 2001. Lipid class dynamics during development in early life stages of shortbelly rockfish and their application to condition assessment. Journal of Fish Biology 58, 1010–1024

Oliva J., F. Bardag-Gorce, J. Li, B. A. French, S.K. Nguyen, S.C. Lu & S.W. French, 2009.Betaine prevents Mallory–Denk body formation in drug-primed mice by epigenetic mechanisms. Experimental and Molecular Pathology, 86:77–86.

Oliveira, M., Pachecho, M., Santos, M.A., 2008. Organ specific antioxidant responses in golden grey mullet (*Liza aurata*) following a short-term exposure to phenanthrene. Science of the Total Environment 396, 70–78.

OSPAR 2008. 2007/2008 CEMP Assessment: Trends and concentrations of selected hazardous substances in sediments and trends in TBT-specific effects. OSPAR Commission publication 378/2008. 36pp

Pacheco, M. et M.A. Santos. 2002. Biotransformation, genotoxic, and histopathological effects of environmental contaminants in European eel (*Anguilla anguilla L.*). *Ecotoxicology and Environmental Safety*, 53: 331-347.

Pajares, M. et D. Pérez-Sala. 2006. «Betaine homocysteine S-methyltransferase: just a regulator of homocysteine metabolism?». *Cellular and Molecular Life Sciences*, 63: 2792-2803.

Peck M.R., Labadie P., Minier C., Hill E.M. 2007. Profiles of environmental and endogenous estrogens in the zebra mussel *Dreissena polymorpha*. Chemosphere. 69:1-8.

Peck, M.; Gibson, R. W.; Kortenkamp, A.; Hill, E. M., Sediments are major sinks of steroidal estrogens in two United Kingdom rivers. 2004, *23*, (4), 945-952.

Pelissero C, Flouriot G, Foucher JL, Bennetau B, Dunoguès J, Le Gac F, Sumpter JP. 1993. Vitellogenin synthesis in cultured hepatocytes; an *in vitro* test for the estrogenic potency of chemicals. J Steroid Biochem Mol Biol 44(3):263-272.

Plante S., C. Audet Y. Lambert de la Noüe J. 2005. Alternative Methods for Measuring Energy Content in Winter Flounder. *North American Journal of Fisheries Management*, 25: 1-6.

Pollux BJA, Korosi A. 2010. Use of stream mouth habitats by *Cottus perifretum* and *Leuciscus cephalus* along the River Meuse (the Netherlands). Folia Zoologica 59 (1):44-50.

Priou F., Barbieri M. 2012. L'évolution démographique récente en France : une mortalité relativement faible aux grands ages. *Population*, 67(4) : 597-656.

Quillardet P., Huisman O., D'Ari R. & Hofnung M. 1982. SOS Chromotest, a direct assay of induction of an SOS function in Escherichia coli K12 to measure genotoxicity. Proceeding of the National. Academy of Sciences USA, 79, 5971-5975.

Rabilloud T., M. Chevallet, S. Luche & C. Lelong, 2010. Two-dimensional gel electrophoresis in proteomics: Past, present and future. Journal of proteomics 73(11):2064-2077. doi:10.1016/j.jprot.2010.05.016.

Randák T, Zlábek V, Kolárová J, Svobodová Z, Hajslová J, Siroká Z, Jánská M, Pulkrabová J, Cajka T, Jarkovský J. 2006. Biomarkers Detected in Chub (*Leuciscus cephalus*; L.) to Evaluate Contamination of the Elbe and Vltava Rivers, Czech Republic. Bulletin of Environmental Contamination and Toxicology 76 (2):233-241.

Rasmussen TH, Teh SJ, Bjerregaard P, Korsgaard B. 2005. Anti-estrogen prevents xenoestrogen-induced testicular pathology of eelpout (*Zoarces viviparus*). Aquatic Toxicology 72 (3):177-194.

Reyjol Y, Lena JP, Hervant F, Pont D. 2009. Effects of temperature on biological and biochemical indicators of the life-history strategy of bullhead *Cottus gobio* (vol 75, pg 1427, 2009). Journal of Fish Biology 75 (10):2880-2880.

Ridgway, J., Breward, N. , Langston, W. J. , Lister, R., Rees J. G.and Rowlatt S. M. 2003 Distinguishing between natural and anthropogenic sources of metals entering the Irish Sea. Applied Geochemistry 18, 283-309

Rocha Monteiro P.R., Reis-Henriques M.A., Coimbra J. 2000. Polycyclic aromatic hydrocarbons inhibit in vitro ovarian steroidogenesis in the flounder (*Platichthys flesus L.*), Aquat. Toxicol, 48:549–559.

Rodríguez-Rúa, A., Prado, M., Romero, Z. & Bruzón, M. 2003. The gametogenic cycle of Scrobicularia plana (da Costa, 1778) (Mollusc: Bivalve) in Guadalquivir estuary (Cádiz, SW Spain). Aquaculture 217, 157-166.

Rolland M., Le Moal J., Wagner V., Royère D., De Mouzon J. 2013. Decline in semen concentration and morphology in a sample of 26 609 men close to general population between 1989 and 2005 in France. *Human Reproduction*, 28 (2): 462-470.

Rose W.L., Nisbet, R.M., Green, P.G., Norris, S., Fan, T., Smith, E.H., Cherr, G.N., Anderson S.L. 2006. Assessment of seasonal variability of biomarkers in three-spined stickleback (*Gasterosteus aculeatus* L.) from a low contaminated stream: Implication for environmental biomonitoring. Aquatic Toxicology 80, 298–308.

Rostkowski, P.; Horwood, J.; Shears, J. A.; Lange, A.; Oladapo, F. O.; Besselink, H. T.; Tyler, C. R.; Hill, E. M., Bioassay-Directed Identification of Novel Antiandrogenic Compounds in Bile of Fish Exposed to Wastewater Effluents. *Environ. Sci. Technol.* 2011, *45*, (24), 10660-10667.

Roubal WT, Lomax DP, Maryjean L, Lyndal LJ. 1997. Purification and partial characterization of English sole (*Pleuronectes vetulus*) vitellogenin. Comp Biochem Physiol **118B**:613-622.

Rowe, C.L. 2003. Growth responses of an estuarine fish exposed to mixed trace elements in sediments over a full life cycle. Ecotoxicology and Environmental Safety 54, 229–239.

Ruiz, J.M., Barreiro, R. and González, J.J. 2005. Biomonitoring organotin pollution with gastropods and mussels. Marine Ecology Progress. 287; 169-176.

Saborido-Rey, F., Dominguez-Petit, R., Tomas Morales-Nin, B., Alonso-Fernandez, A., 2007. Growth of juvenile turbot in response to food pellets contaminated by fuel oil from the tanker 'Prestige'. Marine Ecology Progress Series 245, 271–279.

Sanchez W, Ait-Aissa S, Palluel O, Ditche JM, Porcher JM. 2007. Preliminary investigation of multi-biomarker responses in three-spined stickleback (*Gasterosteus aculeatus* L.) sampled in contaminated streams. Ecotoxicology 16 (2):279-287.

Sanchez W, Goin C, Brion F, Olsson PE, Goksoyr A, Porcher JM. 2008a. A new ELISA for the three-spined stickleback (*Gasterosteus aculeatus* L.) spiggin, using antibodies against synthetic peptide. Comp Biochem Physiol C-Toxicol Pharmacol 147 (1):129.

Sanchez W, Katsiadaki I, Piccini B, Ditche JM, Porcher JM 2008b. Biomarker responses in wild three-spined stickleback (*Gasterosteus aculeatus* L.) as a useful tool for freshwater biomonitoring: A multiparametric approach. Environment International 34 (4):490-498.

Sanchez W, Sremski W, Piccini B, Palluel O, Maillot-Marechal E, Betoulle S, Jaffal A, Ait- Aissa S, Brion F, Thybaud E, Hinfray N, Porcher J-M. 2011. Adverse effects in wild fish living downstream from pharmaceutical manufacture discharges. Environment International 37 (8):1342-1348

Sanchez W., Palluel O., Meunier L., Coquery M., Porcher J.M., Aït-Aïssa S. 2005. Copper-induced oxidative stress in three-spined stickleback: relationship with hepatic metal levels. Environmental Toxicology and Pharmacology 19, 177–83.

Sanchez W., Piccini B., Ditche J.M., Porcher J.M., 2008. Assessment of seasonal variability of biomarkers in three-spined stickleback (*Gasterosteus aculeatus* L.) from a low contaminated stream: Implication for environmental biomonitoring. Environment International 34, 791–798.

Sanchez, B. C., K. J. Ralston-Hooper, K. A. Kowalski, H. Dorota Inerowicz, J. Adamec & M. S. Sepulveda, 2009. Liver proteome response of largemouth bass (*Micropterus salmoides*) exposed to several environmental contaminants: potential insights into biomarker development. Aquatic Toxicology 95(1):52-59.

Santos, M.M., Viera, N. Reis-Henriques, M.A., Santos, A.M., Gomez-Ariza, J.L., Giraldaez, I. and ten Hallers-Tjabbes, C.C 2004. Imposex and butyltin contaminants off the Oporto Coast (NW Portugal): a possible effect of the discharge of dredged material. Environment International. 30; 793-798.

Schlenk D., Perkins E.J., Hamilton G., Zhang Y.S., Layher W. 1996. Correlation of hepatic biomarkers with whole animal and population.community metrics. Canadian Journal of Fisheries and Aquatic Sciences 56, 2299–2308.

Segner H. 2009. Zebrafish (*Danio rerio*) as a model organism for investigating endocrine disruption. Comp Biochem Physiol C Toxicol Pharmacol, 149(2):187-195.

Segner H., Caroll K., Fenske M., Janssen C.R., Maack G., Pascoe D., Schäfers C., Vandenbergh G.F., Watts M., Wenzel A., 2003. Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. Ecotoxicol Environ Saf, 54(3):302-314.

Selleslagh J.R., Amara R., Laffargue P., Lesourd S., Lepage M., Girardin M. 2009. Composition and functioning of three fish estuarine assemblages of the eastern English Channel: a comparison with French estuaries. Estuarine, Coastal and Shelf Science, 81, 149–159.

Sherry J, Gamble A, Fielden M, Hodson P, Burnison B, Solomon K. 1999. An ELISA for brown trout (*Salmo trutta*) vitellogenin and its use in bioassays for environmental estrogens. Science of the Total Environment 225 (1-2):13-31.

Shilling A.D., Carlson D.B., Williams D.E. 1999. Rainbow trout, *Oncorhynchus mykiss*, as a model for aromatase inhibition. J. Steroid Biochem. Mol. Biol, 70:89–95.

Skouras A., Broeg K., Dizer H., von Westernhagen H., Hansen P.D, Steinhagen D.. 2003. The use of innate immune responses as biomarkers in a programme of integrated biological effects monitoring on flounder (*Platichthys flesus*) from the southern North Sea. *Helgoland Marine Research*, 57: 190-198.

Smeets JMW, Rankouhi TR, Nichols KM, Komen H, Kaminski NE, Giesy JP, Van den Berg M. 1999a. In VitroVitellogenin Production by Carp (*Cyprinus carpio*) Hepatocytes as a screening method for determining (anti)estrogenic activity of xenobiotics. Toxicol Appl Pharmacol 157(1):68-76.

Smith, C.A., T. Farrah et R.G. Goodwin. 1994. The TNF receptor superfamily of cellular and viral proteins: Activation, costimulation, and death. *Cell*, 76: 959-962.

Sola, J. 1997. Reproduction, population dynamics, growth and production of Scrobicularia plana da Costa (pelecypoda) in the Bidasoa estuary, Spain. Netherlands Journal of Aquatic Ecology 30, 283-296.

Solé M., Raldua D., Piferrer F., Barceló D., Porte C. 2003. Feminization of wild carp, *Cyprinus carpio*, in a polluted environment: plasma steroid hormones, gonadal morphology and xenobiotic metabolizing system. Comp Biochem Physiol C Toxicol Pharmacol, 136(2):145-156.

Stentiford GD, Longshaw M, Lyons BP, Jones G, Green M, Feist SW. 2003. Histopathological biomarkers in estuarine fish species for the assessment of biological effects of contaminants. Mar Environ Res, 55: 137-159.

Sumpter J. P., Jobling S. 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ Health Perspect, 103(7):173–178.

Sumpter J.P. 2002. Endocrine disruption in the aquatic environment. In: The Handbook of Environmental Chemistry Vol. 3, Part M, Endocrine Disruptors, Part II (ed. by M. Metzler) Springer-Verlag Berlin Heidelberg.

Sumpter JP. 1998. Xenoendocrine disrupters - environmental impacts. Toxicology Letters 103:337-342.

Tahara D, Hatano R, Iwatani H, Koya Y, Hayakawa Y. 2010. Annual changes in testicular development and occurrence of parasperm in the male reproductive organs of fourspine sculpin, Cottus kazika. Ichthyological Research 57 (1):62-70.

Thain JE, Vethaak AD, Hylland K. 2008. Contaminants in marine ecosystems: developing an integrated indicator framework using biological-effect techniques. ICES J Mar Sci 65: 1508-1514.

Thévenot, D., Meybeck, M., Chesterikov, A., Chevreuil, M., 1998. In: Meybeck M, de Marsily G, Fustec E, editors. Métaux : sources multiples et accumulation, La Seine en son bassin, Fonctionnement écologique d'un système fluvial anthropisé, Chap 9. Elsevier, Paris, 391- 437.

Trubiroha A., Kroupova H., Wuertz S., Frank S.N., Sures B., Kloas W. 2010. Naturally-induced endocrine disruption by the parasite *Ligula intestinalis* (Cestoda) in roach (*Rutilus rutilus*). Gen Comp Endocrinol, 166(2):234-40.

Tutundjian R, Minier C, LeFoll F, Leboulenger F. 2002. Rhodamine exclusion activity in primary cultured Turbot (*Scophthalmus maximus)* hepatocytes. Mar Environ Res 54:443-447.

Tyler C. R., Sumpter, J. P., 1996. Oocyte growth and development in teleosts. Fish Biol Fisheries, 6(3):287-318.

Tyler C.R., Jobling, S., Sumpter, J.P. 1998. Endocrine disruption in wildlife: a critical review of the evidence. Crit Rev Toxicol. 28(4):319-361.

Tyler C.R., Lange, A., Paull, G.C., Katsu, Y., Iguchi, T., 2007. The roach (*Rutilus rutilus*) as a sentinel for assessing endocrine disruption. Environ Sci, 14(5):235-253.

Tyler C.R., Pottinger, T.G., Santos, E., Sumpter, J.P., Price, S.A., Brooks, S., Nagler, J.J., 1996. Mechanisms controlling egg size and number in the rainbow trout, *Oncorhynchus mykiss*. Biol Reprod, 54(1):8-15.

Tyler CR, van Aerle R, Hutchinson TH, Maddix S, Trip H. 1999. An *in vivo* testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. Environmental Toxicology and Chemistry 18 (2):337-347.

Urbatzka, R.; van Cauwenberge, A.; Maggioni, S.; Vigano, L.; Mandich, A.; Benfenati, E.; Lutz, I.; Kloas, W., Androgenic and antiandrogenic activities in water and sediment samples from the river Lambro, Italy, detected by yeast androgen screen and chemical analyses. *Chemosphere*. 2007, *67*, (6), 1080-1087.

van Aerle R, Nolanusan M, Jobling S, Christiansen LB, Sumpter JP, Tyler CR. 2001. Sexual disruption in a second species of wild cyprinid fish (the gudgeon, *Gobio gobio*) in United Kingdom Freshwaters. Environmental Toxicology and Chemistry 20 (12):2841-2847.

Van den Belt K, Wester PW, Van der Ven LTM, Verheyen R, Witters H. 2002. Effects of ethynylestradiol on the reproductive physiology in zebrafish (*Danio rerio*): Time dependency and reversibility. Environmental Toxicology and Chemistry 21 (4):767- 775.

Van Der Kraak G.J., Munkittrick K.R., McMaster M.E., Portt C.B., Chang J.P. 1992. Exposure to bleached kraft pulp mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. *Toxicol*. Appl. Pharmacol, 115: 224-233.

Van der Ven LTM, Wester PW, Vos JG. 2003. Histopathology as a tool for the evaluation of endocrine disruption in zebrafish (*Danio rerio*). Environmental Toxicology and Chemistry 22 (4):908-913.

Vandegehuchte M. B,. Janssen C. R. 2011. Epigenetics and its implications for ecotoxicology. Ecotoxicology 20(3):607-624.

Vethaak AD, Wester PW (1996) Diseases of flounder Platichthys flesus in Dutch coastal and estuarine waters, with particular reference to environmental stress factors. II. Liver histopathology. Dis Aqua Org, 26: 99-116.

Vinggaard, A. M.; Niemela, J.; Wedebye, E. B.; Jensen, G. E., Screening of 397 chemicals and development of a quantitative structure-activity relationship model for androgen receptor antagonism. *Chem*. *Res*. *Toxicol*. 2008, *21*, (4), 813-823.

Vos JG, Dybing E, Greim HA, Ladefoged O, Lambre C, Tarazona JV, Brandt I, Vethaak AD (2000) Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. Critical Reviews in Toxicology 30 (1):71-133.

Walker C, Kaiser K, Klein W, Lagadic L, Peakall D, Sheffield S, Soldan T, Yasuno M. 1998. 13th Meeting of the scientific group on methodologies for the safety evaluation of chemicals (SGOMSEC): alternative testing methodologies for ecotoxicity. Environ Health Perspect 106:441-451.

Wang J., Y. Wei D. Wang L.L. Chan J. Dai. 2008. Proteomic study of the effects of complex environmental stresses in the livers of goldfish (*Carassius auratus*) that inhabit Gaobeidian Lake in Beijing, China. Ecotoxicology, 17(3):213–220.

Wei Y., Chan L.L., Wang D., Zhang H., Wang J., Dai J. 2008. Proteomic analysis of hepatic protein profiles in rare minnow (*Gobiocypris rarus*) exposed to perfluorooctanoic acid. Journal of Proteome Research 7:1729–1739.

Weiss, J. M.; Hamers, T.; Thomas, K. V.; van der Linden, S.; Leonards, P. E. G.; Lamoree, M. H., Masking effect of anti-androgens on androgenic activity in European river sediment unveiled by effect-directed analysis. *Anal*. *Bioanal*. *Chem*. 2009, *394*, (5), 1385-1397.

Williams T. D., Diab A., Ortega F., Sabine V. S., Godfrey R. E., Falciani F., Chipman J. K., George S. G. 2008. Transcriptomic responses of European flounder (*Platichthys flesus*) to model toxicants. Aquat Toxicol 90(2):83-91 doi:10.1016/j.aquatox.2008.07.019.

Wirgin I et al. 2011. Mechanistic basis of resistance to PCBs in Atlantic tomcod from the Hudson river. Science, vol. 331: 1322-1325.

Wirgin I., Waldman J.R.. 1998. Altered gene expression and genetic damage in North American fish populations. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis*, 399: 193-219.

Woo S., Yum S. 2011. Transcriptional response of marine medaka (*Oryzias javanicus*) on exposure to toxaphene. *Comparative Biochemistry* and Physiology Part *C*: *Toxicology & Pharmacology* 153:355–361.

Xu W., I.G. Charles et S. Moncada. 2005. «Nitric oxide: orchestrating hypoxia regulation through mitochondrial respiration and the endoplasmic reticulum stress response». *Cell Res*, 15 : 63-65.

Yum S., S. Woo E. Choi S. Kim R. Oh S. Lee, Lee T.K.. 2005. Analysis of Gene Expression in Benzo[a]pyrene-exposed Sebastes schlegeli using Differential Display Polymerase Chain Reaction. *J Environ Toxicol*, vol. 20, no 1, p. 67-73.

Yunker M.B., MacDonald R.W., Goyette D., Paton D.W., Fowler B.R., Sullivan D., Boyd J. 1999. Natural and anthropogenic inputs of hydrocarbons to the strait of Georgia. *Sci*. *Total Environ*., 225 : 181-209.

Zanuy S, Carrillo M, Mateos J, Trudeau V, Kah O. 1999. Effects of sustained administration of testosterone in pre-pubertal sea bass (*Dicentrarchus labrax* L). Aquaculture 177 (1- 4):21-35.

Zelikoff J.T. 1998. Biomarkers of immunotoxicity in fish and other non-mammalian sentinel species: predictive value for mammals?. *Toxicology*, vol. 129, no 1, Aug, p. 63-71.

Ziv T., T. Gattegno, V. Chapovetsky, H. Wolf, E. Bamea, E. Lubzens & A. Admon, 2008. Comparative proteomics of the developing fish (zebrafish and gilthead seabream) oocytes. Comparative Biochemistry and Physiology Part D, 3:12–35.

VII. COMMUNICATION OF RESULTS

VII.1. General description

In accordance with the objectives of the DIESE programme, partners of the project have invested a lot of their time to exchange on the results obtained during the programme. This includes discussions between scientists during meetings, samplings and common laboratory experiments. Results and generated knowledge were then largely disseminated to:

- public institutions during specific meeting organised by the DIESE partners or organised by other collaborators ;
- fishermen during samplings exercises;
- industrials and other end-users during meetings;
- general public in the course of open meetings and television interviews;
- University students as the results are used to illustrate built special courses;
- scientific community in national and international congress.

As one of the specific objectives of the DIESE programme was to contribute to the planning and implementation of specific tools to manage the aquatic environment, regular meetings took place with representatives of the ONEMA, Agence de l'Eau and Environmental Agency. Transfer of the intersex clam tool has been operated during two days of demonstration, sampling and exchanges between researchers from the Marine Biological Association, the University of Le Havre and people in charge of the management of the Natural reserve of the Saint Brieuc Bay (July 23-24, 2012). Measurements were conducted by the people of the natural reserve in 2013.

VII.2. Communications to the general public

VII.2.1. Meetings open or dedicated the general public

- Perturbateurs endocriniens et biodiversité. Colloque du Réseau Environnement Santé et World Wide Fund for Nature. MNHN, 28 avril 2011.
- Chemical contaminants and the sexual chemistry of fish and fœtal health. Open lecture for the general public and staff. University of Sussex, May 2012.
- Qualité des eaux et santé des organismes : Quels composés biologiquement actifs ? Quels effets sur le système hormonal, sur le système immunitaire ? Réunion de restitution du programme Européen DIESE, Le Havre, 5 juin 2013.

VII.2.2. TV emissions

« Une rivière de médicaments », Global Mag, Arte, 19 juin 2009

« Estuaire de la Seine, une pollution invisible », Thalassa (France 3), mai 2009

« Du poison dans l'eau du robinet », France 3, 17 mai 2010

« Du poison dans l'eau du robinet », Les documentaires France 3, 27 aout 2013

« Le Zapping » Canal plus, 30 aout 2013

VII.3. Scientifiques communications

VII.3.1. Oral communications in scientifiques congress

Fifty-seven oral communications and numerous poster presentations (not listed here) were realised in scientific congress.

Calves I., C. Dupuy, E. Lavergne, C. Galland, C. Capitaine, N. Pédron, G. Charrier, B. Guinand, L. Quiniou, J. Laroche. 2011. Structure génétique et réponses aux multi-stress de populations de flet (*Platichthys flesus*) sur un cline latitudinal. ECOBIM 2011: Atelier international sur l'évaluation du stress environnemental (Effets biologiques des contaminants chimiques chez les bivalves et les poissons). Institut Maurice-Lamontagne, Mont-Joli, Québec, 6-9 juin 2011.

Calves I., Lavergne E, Leïla Meistertzheim A, Charrier G, Cabral H, Guinand B, Quiniou L, Laroche J. Genetic structure of the European flounder (*Platichthys flesus)* considering the southern limit of the species' range and the potential impact of chemical stress. *8th International Flatfish Symposium, Ijmuiden, The Nederlands, 5-11 novembre 2011*.

Ciocan Corina M., 2012. Reference gene selection for qPCR in mussels – will we ever find a "universal housekeeping gene"? Presentation given by Dr. Corina Ciocan at the University of Le Havre, February 2012.

Ciocan Corina M., Elena Cubero Leon, Jeanette Rotchell -2011. Using new molecular techniques to highlight cellular pathways affected by endocrine disruption. Oral presentation, Interreg IV DIESE CRONEXPO Joint Meeting Caen 2011.

Ciocan Corina M., Elena Cubero-Leon, Keith Cornelius, Mika Peck, William J. Langston, Nick Pope, Christophe Minier and Jeanette M. Rotchell. 2013. Proliferating cell nuclear antigen in the gonad of *Scrobicularia plana*: molecular cloning and expression pattern in clams from natural populations. Poster Presentation, PRIMO 17, Algarve, Portugal.

Ciocan Corina M., Elena Cubero-Leon, Liz Hill, Diana Alvarez Munoz , Mika Peck , Keith Cornelius , William J. Langston, Nick Pope, Christophe Minier and Jeanette M. Rotchell. 2013. Using expression of testes specific genes to assess the effects of environmental endocrine disruptors in gonads of clams (*scrobicularia plana*) from contaminated coastal areas. Platform presentation, 7th International Conference on Marine Pollution and Ecotoxicology, Hong Kong.

Ciocan Corina M., Elena Cubero-Leon, William J. Langston, Nick Pope, Christophe Minier , Frauke Seemann and Jeanette M. Rotchell. Intersexuality in *Scrobicularia plana*: transcriptomic analysis reveals novel genes involved in endocrine disruption. Poster presentation, SETAC 2012, Berlin.

Ciocan Corina M., Elena Cubero-Leon, William J. Langston, Nick Pope, Christophe Minier and Jeanette M. Rotchell. Molecular cloning and characterization of several sex related genes in the bivalve *Scrobicularia plana* (Veneroida). Platform presentation, ESCPB Congress 2012, Bilbao, Spain.

Ciocan Corina, 2013. Mechanisms of endocrine disruption in molluscs. Oral presentation, Final Diese meeting, Le Havre, France June 5th.

Dupuy C, Auffret M, Calves I, Claireaux G, Théron M, Amerand A, Galland C, Guérard F, Loizeau V, Quiniou L, Sanchez W, Fournier M, Laroche J. (2011). Etude de populations naturelles de flets le long d'un gradient latitudinal : biomarqueurs aux niveaux cellulaire, biochimique et physiologique. Colloque d'Immunotoxicologie, Armand-Frappier, 17-19 novembre 2011, Esterel, Québec (Canada)

Dupuy C, Calvès I, Pedron N, França S, Vasconcelos RP, Auffret M, Quiniou L, Laroche J. (2012). Cellular and molecular responses of the European flounder *(Platichthys flesus)* to the effects of climate change, hypoxia and chemical stress, in several estuaries from France to Portugal. 28th Congress – European Society for Comparative Physiology and Biochemistry – Bilbao, 2-5 September 2012.

Dupuy C., C. Galland, I. Calves, M. Auffret, M. Labonne, L. Quiniou, V. Pichereau, J. Laroche. 2011. Responses of flounder populations to multi-stress in estuaries: Immunotoxicity, biochemical biomarkers, Isotopes, Life-history traits and population genetics. Joint meeting CHRONEXPO/DIESE at the University of Caen, INTERREG IV. 30-31 August 2011.

Dupuy C., C. Oudard, M. Auffret, M. Labonne, E. Morize, L. Quiniou & J. Laroche, 2010. Integration of physiological, immunotoxic and genetic responses of the flounder *Platichthys flesus*, in contaminated estuaries. Second Annual Steering Committee. DIESE Project, INTERREG IVA, Plymouth: 17th may 2010

Dupuy C., I. Calves, W. Sanchez, M. Fournier, M. Auffret, L. Quiniou, J. Laroche. 2011. Réponses aux stress chimiques de deux espèces sentinelles de la qualité des milieux estuariens en France et au Québec : le flet et le poulamon. Colloque du GDR EXECO, Arcachon : 7-8 avril 2011.

Dupuy C., M. Auffret, I. Calves, G. Claireaux, M. Théron, A. Amérand, C. Galland, F. Guérard, V. Loizeau, L. Quiniou, W. Sanchez, M. Fournier, J. Laroche. 2011. Etude de populations naturelles de flets le long d'un gradient latitudinal : biomarqueurs aux niveaux cellulaire, biochimique et physiologique. ECOBIM 2011: Atelier international sur l'évaluation du stress environnemental (Effets biologiques des contaminants chimiques chez les bivalves et les poissons). Institut Maurice-Lamontagne, Mont-Joli, Québec, 6-9 juin 2011.

Galland C, Dupuy C, Capitaine C, Claves I, Auffret M, Quiniou L, Laroche J, Pichereau V (2011). Comparisons of liver proteomes in European flounder *Platichthys flesus* from three contrasted estuaries. *8th International Flatfish Symposium, Ijmuiden, The Nederlands, 5-11 novembre 2011*.

Galland C, Dupuy C, Quiniou L, Auffret M, Laroche J, Pichereau V. (2012). Response of the European flounder *Platichthys flesus* to experimental and *in situ* contaminations: a proteomic approach. 28th Congress – European Society for Comparative Physiology and Biochemistry – Bilbao, 2-5 September 2012.

Galland C. Réponse du flet Européen *Platichthys flesus* à la contamination chimique : approche protéomique. Colloque de restitution final, INTERREG IVA DIESE, Le Havre, 5 juin 2013.

Galland C., C. Dupuy, C. Capitaine, I. Calves, M. Auffret, L. Quiniou, J. Laroche, V. Pichereau. 2011. Proteomic analysis of the European flounder *Platichthys flesus* response to HAP/PCB contamination. SETAC EUROPE Annual Meeting, Milan, Italy, 15-19 May 2011.

Galland C., C. Dupuy, C. Capitaine, I. Calves, M. Auffret, L. Quiniou, J. Laroche, V. Pichereau. 2011. Réponse du flet européen *Platichthys flesus* à la contamination par les HAP/PCB : approche par électrophorèse 2D. ECOBIM 2011: Atelier international sur l'évaluation du stress environnemental (Effets biologiques des contaminants chimiques chez les bivalves et les poissons). Institut Maurice-Lamontagne, Mont-Joli, Québec, 6-9 juin 2011.

Géraudie P., Gerbron M., Hinfray N., Brion F., Rotchell J., Hill E.M., Minier C. Steroidogenesis in wild roach (*Rutilus rutilus*): alterations by environmental pollutants. PRIMO 15 symposium (Pollutant responses in marine organisms). Bordeaux May 17-20, 2009.

Geraudie P., Nahrgang J., Marie G., Minier C., Camus L. Is produced water able to alter endocrine function and reproduction in polar cod (*boreogadus saida*) during chronic exposure with realistic environmental concentrations? PRIMO 16 symposium (Pollutant responses in marine organisms). Long Islands, CA, May 15-18, 2010.

Gerbron M., P. Geraudie, B. Xuereb, E.M. Hill, J. R. Rotchell, C. Minier. Combined effect of cadmium and estradiol on the endocrine system of roach (*Rutilus rutilus*): *In vitro* and *in vivo* approaches. ESCBP meeting, 2-5 September, 2012, Bilbao, Spain.

Gerbron M., P. Geraudie, B. Xuereb, J. R. Rotchell, C. Minier. *In vitro* and *in vivo* determination of the Cadmium interactions with the endocrine system of Roach *(Rutilus rutilus)*, 16th International Symposium on Pollution Responses in Marine Organisms PRIMO16, 15-18 May 2011, Long Beach, California, USA

Hill E.M. Are we living in a world contaminated with antiandrogenic chemicals? NORMAN network workshop, Amsterdam, November 2012.

Hill E.M., Al-Sahli, R.A., Abdul-Sada, A., Lange, A., and Tyler, C.R. The xenometabolome and novel contaminant markers in fish exposed to a wastewater treatment works effluent. 28th Congress European Society for Comparative Physiology and Biochemistry – Bilbao, 2-5 September 2012.

Hill E.M., Cubero E., Rotchell J.M., Minier C., Flores-Valverde A.M. Investigating pathways of aquatic toxicity of environmental estrogens using metabolomic analyses. PRIMO 15 symposium (Pollutant responses in marine organisms). Bordeaux May 17-20, 2009.

Kerambrun, E., Henry, F., Cornille, V., Courcot, L., and Amara, R. Metal bioconcentrations and condition indices in juvenile European flounders, *Platichthys flesus*, from European estuaries. 50th Estuarine and Coastal Sciences ECSA 50, 3-7 June 2012, Venice, Italy.

Kerambrun, E., Henry, F., Sanchez, W., Gevaert, F., Spilmont, N. and Amara, R. Physiological performance of caged juvenile sea bass (*Dicentrarchus labrax*) and turbot (*Scophtalmus maximus*) in a contaminated area. 47th Estuarine and Coastal Sciences ECSA 47, 14-19 September 2010, Figueira Da Foz, Portugal.

Kerambrun, E., Le Floch, S., Thomas-Guyon, H., Sanchez, W. and Amara, R. Etude des relations entre les réponses de biomarqueurs et les performances physiologiques de juvéniles de bar commun, *Dicentrarchus labrax* exposés à une contamination au pétrole. 25è Forum des jeunes océanographes, 28-29 octobre 2009, La Rochelle, France.

Kerambrun, E., Perrichon, P., Henry, F., Sanchez, W., and Amara, R. Influence de sédiments contaminés sur les performances physiologiques et les réponses de biomarqueurs de juvéniles de turbot *Scophtalmus maximus*. 26è Forum des jeunes océanographes, 30 septembre et 1er octobre 2010, Wimereux, France.

Kerambrun, E., Perrichon, P., Henry, F., Sanchez, W., Courcot, L. and Amara R. Growth, condition indices and biomarker responses of juvenile turbot, *Scophtalmus maximus*, exposed to contaminated sediment. 16th International Symposium on Pollution Responses in Marine Organisms PRIMO16, 15-18 May 2011, Long Beach, California, USA.

Labonne M, Calves I, Dupuy C, Claireaux G, Amérand A, Théron M, Loizeau V, Dabas E, Munaron JM, Cabral H, Vasconcelos RP, França S, Quiniou L, Laroche J (2012). Réponses phénotypiques du flet (*Platichthys flesus*) dans des estuaires à environnements contrastés, sur un cline Nord-Sud. *Rencontre de l'Ichtyologie en France, Paris, 27-30 mars 2012*.

Langston WJ, Pope ND. Endocrine Disruption (ED) in *Scrobicularia plana*. Shellfish Association, Fishmongers Hall, London, Feb 2010.

Langston WJ. Pollution indicators. MBA annual science conference. Plymouth, April 2012.

Laroche J, Calves I, Claireaux G, Amérand A, Théron M, Loizeau V, Labonne M, Sanchez W, Cabral H, Vasconcelos RP, França S, Pedron N, Quiniou L (2012). Climate change, hypoxia and chemical stress : Impacts on the energetic metabolism of the European flounder *Platichthys flesus*. Colloque franco-Québécois ECOBIM, Reims 5-8 juin 2012.

Laroche J, Dupuy C. (2013). Intégration des réponses moléculaires, biochimiques et immunotoxicologiques face aux contaminants. Colloque de restitution final, INTERREG IVA DIESE, Le Havre, 5 juin 2013.

Laroche J, Marchand J, Quiniou L. (2013). Relations genotypes-phénotypes dans des populations de flet (*Platichthys flesus)* exposées à des contaminations différentielles en estuaries. Colloque franco-Québécois ECOBIM, Montréal, 27-30 mai 2013.

Laroche J. (2013). Approche intégrative en écotoxicologie sur un poisson estuarien, le flet (*Platichthys flesus)*. Colloque de l'ARET (Association de Recherche en Toxicologie et Ecotoxicologie), Paris, 20-21 juin 2013.

Laroche J., I. Calves, C. Dupuy, C. Galland, M. Auffret, V. Pichereau, C. Capitaine, M. Labonne, E. Dabas, J.M. Munaron, F. Guérard, L. Quiniou, G. Claireaux, M. Théron, A. Amérand, V. Loizeau, D. Mazurais, J. Zambonino, N. Le Bayon, H. Cabral, R. Vasconcelos, S. Franca, M. Fournier, C. Couillard . 2011. Approche intégrative de la réponse de populations de poissons aux multi-stress en milieu estuarien. ECOBIM 2011: Atelier international sur l'évaluation du stress environnemental (Effets biologiques des contaminants chimiques chez les bivalves et les poissons). Institut Maurice-Lamontagne, Mont-Joli, Québec, 6-9 juin 2011.

Laroche J., L. Quiniou, E. Evrard, A. Devaux, S. Bony 2010. Responses of juvenile European flounder (*Platichthys flesus)* to multi-stress in the Vilaine estuary, during a six-month survey. Second Annual Steering Committee. DIESE Project, INTERREG IVA, Plymouth: 17th may 2010

Laroche J., L. Quiniou, E. Evrard, A. Devaux, S. Bony 2010. Un exemple d'approche intégrative: réponses de juvéniles de flet (*Platichthys flesus)* au multi-stress en estuaire de Vilaine, durant un suivi de 6 mois. ECOBIM 2010: Atelier international sur l'évaluation du stress environnemental (Effets biologiques des contaminants chimiques chez les bivalves et les poissons). Ifremer, Nantes : 18-19 mai 2010.

Maréchal A., Fernandes D., Rotchell JR, Porte C., Minier C. Antiandrogenic effects in roach (*Rutilus rutilus*): an *in-situ* study based on the assessment of male gonadal steroidogenesis activities. ESCBP meeting, 2-5 September, 2012, Bilbao, Spain.

Minier C. Determination of Indicator for Environmental monitoring: a Strategy for Europe. Gagileau Workshop. Bordeaux November, 29-30th, 2010.

Minier C. Effects of xeno-estrogens, androgens and anti-androgens on fish and mollusc from the Seine River. Malang, Brawijaya University (Indonesia), January 2, 2012 International conference on Ecotoxicology.

Minier C. Effects of xeno-estrogens, androgens and anti-androgens on fish, crustaceans and mollusk. Cape Town (South Africa), University of the Western Cape, July 30th, 2012.

Minier C. Endocrine-disrupting effects of anti-androgens on fish and mollusk physiology. Taipei University (Taiwan), September 11th, 2012.

Minier C. Estrogenic, androgenic and anti-estrogenic effects in fish from the French streams. 15th symposium on toxicity assessment (ISTA15). Metz, August 30 – September 4, 2009.

Minier C., Geraudie P., Hinfray N., Porcher J.-M., Brion F., Hill E., Rotchell J. R. Effects of xeno-estrogen and anti-androgens on fish from the seine river. 15th symposium on toxicity assessment (ISTA15). Hong-Kong, July 2–8, 2011.

Minier C., Gerbron M., Geraudie P., Xuereb B., Denier X., Hill E.M., Rotchell J.R. combined effect of cadmium and estradiol on the endocrine system of roach (*Rutilus rutilus*): *In vitro* and *in vivo* studies. 16th symposium on toxicity assessment (ISTA16). Cape Town, February 21–26, 2012.

Minier C., Rotchell J.M., Hill E., Monsinjon T., Knigge T. Endocrine disrupting effects in bivalves. 14th International Symposium on Toxicity Assessment (ISTA14), Metz, August 30 - September 4, 2009.

Pedron N, Lavergne E, Calvès I, Mazurais D, Zambonino J, Le Bayon N, Quiniou L, Laroche J. (2013). Does the chronic chemical contamination of a European flounder population decrease its thermal tolerance? PRIMO 17, Pollutant responses in marine organisms, Algarve, 5th-8th May 2013.

Pope ND, Langston WJ. Water quality and shellfish. Workshop at Cefas Weymouth 8-9th February 2010.

Sanchez W., Villeret M., Piccini B., Minier C., Sremski W., Blanchard C., Porcher J.M. Androgenic contamination of freshwater ecosystems cannot be ignored for environmental risk assessment. 3rd SETAC Europe special science symposium, Brussels, 2-3 February 2011.

Seemann F., Monsinjon T., Knigge T., Minier C. Impact of 17-β estradiol on the development of sea bass immune system. PRIMO 16 symposium (Pollutant responses in marine organisms). Long Islands, CA, May 15-18, 2010.

Seemann F., T. Monsinjon, S. Olivier, T. Knigge, C. Minier. Impairment of juvenile sea bass (*Dicentrarchus labrax*) innate immunity following waterborne 17-β estradiol exposure is reversible. ESCBP meeting, 2-5 September, 2012, Bilbao, Spain.

Seemann F., T. Monsinjon, T. Knigge, C. Minier. Impact of 17-β estradiol on the development of sea bass immune system, 16th International Symposium on Pollution Responses in Marine Organisms PRIMO16, 15-18 May 2011, Long Beach, California, USA.

Wessel N., F. Henaff, V. Buchet, A. Sévère, N. Le Bayon, A. M. Le Guellec, K. Héas-Moisan, C. Munschy, V. Loizeau, C. Minier. Reproductive disruption induced on common sole (Solea solea) following in vivo exposure to polybrominated diphenyl ethers and polychlorinated biphenyls. 16th International Symposium on Pollution Responses in Marine Organisms PRIMO16, 15-18 May 2011, Long Beach, California, USA.

VII32.2. Documents and book chapters

Amara, R., 2011. Impact de la pollution sur les écosystèmes côtiers : exemple de la Manche orientale. *VertigO - la revue électronique en sciences de l'environnement*, Hors série 9. http://vertigo.revues.org/10990.

Minier, C., Amara, R., 2009. From pollution to altered fish biological and physiological performances; the case of flatfish in the Seine estuary. In 'Environmental assessment of estuarine ecosystems: a case study'. Eds: C. Amiard-Triquet and P.S. Rainbow, CRCPress / Taylor and Francis. 344 pp.

Gubbins M.J., Huet M., Reiner M.M., Minier C. 2013. Impairement of endocrine functions : case studies. In Amiard-Triquet C., Amiard J-C. and Rainbow P.S., *Ecological biomarkers*. CRC Press. pp 219-252.

VII.3.3. Publications in scientific journals

Thirty-five manuscripts were submitted and accepted for publication in international scientific journals.

Boulangé-Lecomte C., Géraudie P., Forget-Leray J., Gerbron M. Minier C. 2011. Another brick in the worm: *Ligula intestinalis* manipulates its main host, the Roach (*Rutilus rutilus*), by interfering with brain and gonad aromatase expression. *J. Helminthology*. 85: 339-344.

Calves I., Lavergne E., Meistertzheim A.L., Charrier G., Cabral H., Guinand B., Quiniou L., Laroche J. (2013). Genetic structure of European flounder (*Platichthys flesus*): effects of both the southern limit of the species' range and chemical stress. Marine Ecology Progress Series. 472, 257-273

Ciocan C, Cubero-Leon E., Minier C, Rotchell JM. 2011. Identification of reproduction-specific genes associated with maturation and estrogen exposure in a marine bivalve *Mytilus edulis*. *PlosOne*, 6(7): e22326. doi:10.1371/journal.pone.0022326.

Ciocan C.M., Cubero-Leon E., Puinean A.M., Hill E.M., Minier C., Osada M., Fenlon K., Rotchell J.M. 2010. Effects of estrogen exposure in mussels, *Mytilus edulis,* at different stages of gametogenesis. *Environ. Pollut*. 158:2977-2984.

Corina Ciocan M., William J. Langston, Nick Pope, Keith Cornelius, Christophe Minier and Jeanette Rotchell. 2012. Intersex in *Scrobicularia plana* – transcriptomic analysis reveals novel genes involved in endocrine disruption. *Environmental Science and Technology* 46: 12936-12944.

Cubero-Leon E., Ciocan C. M., Hill E. M., Osada M., Kishida M., Itoh N., Kondo R., Minier C., Rotchell J.M. 2010. Estrogens disrupt *serotonin receptor* and *cyclooxygenase* gene expression in the gonads of mussels (*Mytilus edulis*). *Aquat. Toxicol*. 98: 178-187.

Cubero-Leon E., Minier C., Rotchell J., Hill EM. 2012. Metabolomic analysis of sex specific metabolites in gonads of the mussel, *Mytilus edulis*. *Comp. Biochem. Physiol*. D7. 212-219.

Cubero-Leon E., Puinean AM., Labadie P, Ciocan C, Itoh N, Kishida M, Osada M, Minier C, Hill EM, Rotchell JM. 2012. Two novel CYP3A genes in the marine mussel *Mytilus edulis*: mRNA expression modulation following short-term exposure to endocrine disruptors. *Mar Environ Res*. 74. 32-39.

Cubero-Leon Elena, Corina M. Ciocan, Christophe Minier, Jeanette M. Rotchell. 2012. Reference gene selection for qPCR in mussel, *Mytilus edulis,* during gametogenesis and exogenous estrogen exposure. Environmental Science and Pollution Research 19:2727-2733.

Dupuy C, Couillard C, Laroche J, Brousseau P, Fournier M (2013). A multibiomarker approach on the Atlantic tomcod (*Microgadus tomcod*) in the St. Lawrence Estuary. Environmental Science and Pollution Research. 20: 749-760.

Evrard E, Devaux A, Bony S, Cachot J, Charrier G, Quiniou L, Laroche J. (2013). Responses of juvenile European flounder (*Platichthys flesus*) to multistress in the Vilaine estuary, during a 6-month survey. Environmental Science and Pollution Research. 20: 676-689.

Galland C, Dupuy C, Auffret M, Quiniou L, Laroche J, Pichereau V (2013) Towards tissue proteome maps in the European flounder (*Platichthys flesus*). Proteome Sciences, en révision.

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

Geraudie P., Gerbron M., Hill E., Minier C. 2010. Roach (*Rutilus rutilus*) reproduction: a study of biochemical and histological parameters in a low contaminated site. *Fish. Physiol. Biochem*. 36: 767-777.

Geraudie P., Gerbron M., Minier C. 2010. Seasonal variations and alteration of sex steroid levels during the reproductive cycle of male roach (*Rutilus rutilus*). *Mar. Environ. Res*. 69: S53-S55.

Geraudie P., Hinfray N., Gerbron M., Porcher J.-M., Brion F., Minier C. 2011. Brain cytochrome P450 aromatase activity in roach (*Rutilus rutilus*): seasonal variations and impact of environmental contaminants. *Aquat. Toxicol*. 105: 378-384.

Geraudie P., Lecomte C., Gerbron M., Hinfray N., Brion F., Minier C. 2010. Endocrine effects of the tapeworm *Ligula intestinalis* in its teleost host, the roach (*Rutilus rutilus*). *Parasitology*. 137: 697-704.

Gerbron M., Geraudie P., Rotchell J.M., Minier C. 2010. A new *in vitro* screening bioassay for the ecotoxicological evaluation of the estrogenic responses of environmental chemicals using roach (*Rutilus rutilus*) liver explant culture. *Environ. Toxicol*. 25: 510-516.

Henry, F., Filipuci, I., Billon, G., Courcot, L., Kerambrun, E., and Amara, R., 2012. Metal concentrations, growth and condition indices in European juvenile flounder (*Platichthys flesus*) relative to sediment

contamination levels in four Eastern English Channel estuaries. Journal of environmental monitoring 14, 3211-3219.

Hill, E. M.; Evans, K. L.; Horwood, J.; Rostkowski, P.; Oladapo, F. O.; Gibson, R.; Shears, J. A.; Tyler, C. R. 2010. Profiles and Some Initial Identifications of (Anti)Androgenic Compounds in Fish Exposed to Wastewater Treatment Works Effluents. *Environ. Sci. Technol.*, *44*, (3), 1137-1143.

Hinfray N., Palluel O., Piccini B., Sanchez W., Aït-Aïssa S., Noury P., Gomez E., Geraudie P., Minier C., Brion F., Porcher J.-M. 2010. Endocrine disruption in wild population of chub (*Leuciscus cephalus*) in contaminated French streams. *Sci. Tot. Environ*. 408: 2146-2154.

Kerambrun E., Henry F., Marechal A., Sanchez W., Minier C., Filipuci I., Amara R. 2012. A multi-biomarker approach in juvenile turbot, *Scophthalmus maximus*, exposed to contaminated sediments. *Ecotox. Environ. Safety*. 80; 45-53.

Kerambrun, E., Amara, R., Henry, F, 2013. Effects of food limitation on nine metal concentrations in liver and PAH metabolites in bile of juvenile turbot, *Scophthalmus maximus*, previously exposed to contaminated sediments. Environmental Toxicology and Chemistry (in press).

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

Kerambrun, E., Henry, F., Courcot, L., Gevaert, F. and Amara R., 2012. Biological responses of caged juvenile sea bass (*Dicentrarchus labrax*) and turbot (*Scophthalmus maximus*) in a polluted harbour. Ecological Indicators 19, 161-171.

Kerambrun, E., Henry, F., Perrichon, P., Courcot, L., Meziane, T., Spilmont, N. and Amara, R., 2012. Growth and condition indices of juvenile turbot, *Scophthalmus maximus*, exposed to contaminated sediments: effects of metallic and organic compounds. Aquatic Toxicology 108, 130-140.

Kerambrun, E., Le Floch, S., Sanchez, W., Thomas-guyon, H., Meziane, T., Henry, F. and Amara, R, 2012. Responses of juvenile sea bass, *Dicentrarchus labrax*, exposed to acute concentrations of crude oil, as assessed by molecular and physiological biomarkers. Chemosphere 87, 692-702.

Kerambrun, E., Sanchez, W., Henry, H., and Amara, R., 2011. Are biochemical biomarker responses related to physiological performance of juvenile sea bass (*Dicentrarchus labrax*) and turbot (*Scophthalmus maximus*) caged in a polluted harbor? Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology 154, 187-195.

Langston W.J., Pope N., O'Hara S., Pascoe P., Gibbs P. 2013. Indicators of Endocrine Disruption *MBA Annual report*, 11-12

Langston W.J., Pope N., O'Hara S.C.M. 2012. Indicators of Endocrine Disruption in the English Channel Region. *JMBA*, Global Marine Environment, 15, 26-29.

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

Marchand J, Quiniou L, Laroche J (2013). Relationship between genotypes and phenotypes in natural populations of the European flounder (*Platichthys flesus*) under different types of contamination in estuaries. Journal of Xenobiotics (in press).

Rostkowski, P.; Horwood, J.; Shears, J. A.; Lange, A.; Oladapo, F. O.; Besselink, H. T.; Tyler, C. R.; Hill, E. M.. 2011. Bioassay-Directed Identification of Novel Antiandrogenic Compounds in Bile of Fish Exposed to Wastewater Effluents. *Environ. Sci. Technol.*, *45*, (24), 10660-10667.

Seemann F., Knigge TA., Rocher B., Minier C. Monsinjon T. 2013. 17β-estradiol induces changes in cytokine levels in head kidney and blood of juvenile sea bass (*Dicentrarchus labrax*, L., 1758). *Marine Environmental Res*. 87-88: 44-51.

Villeret M., Jolly S., Wiest L., Vulliet E., Bado-Nilles A., Porcher J.-M., Betoulle S., Minier C., Sanchez W. 2012. A potential biomarker of androgen exposure in European bullhead (*Cottus sp*.) kidney. *Fish Physiol Biochem*. DOI 10.1007/s10695-012-9720-3.

VII.4. Training for young researchers

The DIESE programme allowed to the consortium to employ young researchers during a PhD thesis or for a post-doctoral position.

VII.4.1. List of the theses that were successfully defended

Elena Cubero Leon. 2009. Integrated metabolomic and gene expression study of estrogenic response mechanisms in the marine bivalve *Mytilus edulis*. University of Sussex.

Perrine Géraudie. 2009. Recherche de biomarqueurs de perturbation endocrinienne chez le gardon (*Rutilus rutilus*), intégration des mécanismes moléculaires et écologiques. Université du Havre.

Kerambrun Elodie. 2011. Evaluation of biological effects of chemical contaminants on juvenile marine fish: a multibiomarker approach in experimental and *in situ* conditions. University of Littoral November 2011.

Filipuci Isil. 2011. Effects of environmental stressors on coastal fish: in situ and experimental approaches. University of Littoral September 2011.

Calvès I. 2011. Effets du réchauffement climatique, de l'hypoxie et de la contamination chimique sur les réponses évolutives de populations de flet (*Platichthy flesus*). Thèse de Doctorat, Université de Bretagne Occidentale. 204p.

Dupuy Célie. 2012. Réponses de populations de poissons au stress chimique en milieux estuariens : intégration des réponses moléculaires, biochimiques et immunotoxicologiques. Thèse de Doctorat, Université de Bretagne Occidentale. 213p.

Galland Claire. 2012. Réponses du flet européen *Platichthys flesus* à la contamination chimique : approche protéomique. Thèse de Doctorat, Université de Bretagne Occidentale. 259p.

Villert Mélanie. 2012. Le chabot comme espèce modèle pour l'évaluation des effets des perturbateurs endocriniens. Université de Normandie –ULH.

Seemann Frauke. 2013. Effets de xéno-œstrogènes sur le système immunitaire du bar, *Dicentrarchus labrax*, au cours du développement. Université de Normandie –ULH.

Gerbron Marie. 2013. Development and implementation of a combination of *in vitro* and *in vivo* approaches to study endocrine disruptions in roach *Rutilus rutilus*. Université de Normandie –ULH.

The DIESE programme contributed to two other thesis which not finished at the time of the final report.