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Abstract

The usefulness of fish biomarkers for freshwater biomonitoring is now well recognized, but they still pose several questions to ecotoxicology researchers. The present study, designed to assess the effects of a small city located in an agricultural river basin watershed on sticklebacks living in an adjacent river, underlines the importance of reference selection. Two reference systems were used to analyse responses of a set of biomarkers, including biotransformation enzymes, oxidative stress parameters, neurotoxicity and endocrine disruption end-points, measured in wild sticklebacks electrofished in a contaminated stream. The results showed that the investigated urban pressure disturbed CYP3A activity but also induced hepatic lipoperoxidation and circulating vitellogenine but this result is strongly influenced by the selected reference system. This work therefore demonstrates the need for further research to identify a robust reference system for stickleback biomarker analysis.

Key words : Three-spined stickleback ; Biomarkers ; Fish ; Reference

1. Introduction

A multi-biomarker approach is extensively used to characterize the effects of freshwater, estuarine or marine ecosystem contamination in various organisms including fish (Schmitt et al., 2005; Sanchez et al., 2008a), molluscs (Brooks et al., 2009; Hagger et al., 2009) and crustaceans (Damasio et al., 2008). As proposed by several authors (Allan et al., 2006; Hagger et al., 2006; Sanchez et al., 2009), this tool could be applied in regulatory monitoring regimes such as the surveillance, operational and investigative monitoring networks specified by the Water Framework Directive (WFD, 2000/60/EC; European Council, 2000). Indeed, biomarkers are considered as integrative tools able to provide complementary information to chemical and ecological analyses used for field monitoring and to improve the link between biological effects observed at the community level and monitored chemical concentrations (Sanchez et al., 2008a). But analysis of data from a multi-biomarker approach is complex because of the influence of biotic and abiotic factors on biomarker responses. Also, for the correct use of biomonitoring data, it is crucial to define a reference system which allows a clear distinction between variations caused by natural factors and those caused by anthropogenic impacts (Flammarion and Garric, 1999; Nixon et al., 1996).

The present study was designed to evaluate the ecotoxicological effects of a small city located on an agricultural water basin on wild fish living in adjacent river. To this purpose, a set of complementary biochemical biomarkers, selected to represent several biological processes, was measured in sticklebacks (Gasterosteus aculeatus L.) collected in three locations of the Escrière river, a stream contaminated by urban and agricultural activities. The set of biomarkers used in this work included biotransformation enzymes involved in xenobiotic metabolisation, such as 7-ethoxyresorufin-O-deethylase (EROD), cytochrome P4503A activity (CYP3A) and glutathione-S-transferase (GST), as well as oxidative stress indicators such as glutathione peroxidase (GPx) and lipoperoxidation measured as thiobarbituric reactive

substance content (TBARS), and acetylcholinesterase (AChE) as a neurotoxicity marker. Endocrine disruption biomarkers, including vitellogenin (VTG) in males and spiggin (SPG) in females, were also assessed to characterize exposure to estrogenic and androgenic chemicals, respectively. All recorded data were analysed using two reference systems. In a first stage, a relative reference, frequently used for upstream/downsteam studies (Vigano et al., 1998) or for stream-profile characterization (Machala et al., 2000), was adopted. In this context, data from downstream locations were compared to biomarker responses measured in fish from the upstream site.

In a second stage, biomarker responses measured in fish from the Escrière river were compared to reference values previously established for sticklebacks living in the North of France (Sanchez et al., 2008b). This strategy, using an "absolute" reference, is considered as promising for the evaluation of biomarker responses on a large scale, but requires a rigorous process for the selection of the reference site (Mayon et al., 2007; Sanchez et al., 2008a). The results of this work provide evidence of the advantages and limits of these reference systems for biomarker analysis in an environmental biomonitoring context.

2. Materials and methods

2.1. Sampling design

During autumn 2008, adult three-spined sticklebacks were electrofished (n=10 to 20 fish) at three independent sites on the Escrière river (Figure 1). All sampling stations exhibited a wild stickleback population and were characterized by various anthropogenic pressures. Indeed, site E1, used as a relative reference, is located in an agricultural area upstream of Colleret city (Nord, France). Sites E2 and E3 are positioned within an urbanized and cultivated area, respectively in and downstream of the city.

An “absolute” reference was determined using fish from the Vallon du Vivier site (VDV), as previously described by Sanchez et al. (2008b). To summarize, adult sticklebacks were electrofished every six weeks from April to October 2006 at this site located in the upper area of the water basin and characterized by very low levels of contaminants in water and sediments.

2.2. Characterisation of water and sediment contamination

For all Escrière sampling sites, concentration of cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), nickel (Ni), selenium (Sn) and zinc (Zn) were measured on the dissolved fraction of water by Inductively Coupled Plasma / Mass Spectrometry (ICP/MS) according to the standard NF EN ISO 17294. The limit of detection for heavy metals in water was 0.09 µg/L. A list of twenty five pesticides widely used in the north of France, described in Sanchez et al. (2008b) and including for example alachlor, atrazine, chlorpyriphos, metolachlor and simazine was measured. This list was added to glyphosate and AMPA. All pesticides were determined using a multiresidue analysis on a gas-chromatograph coupled with a mass spectrometer (GS-MS). Prior GC-MS analysis, a liquid-solid extraction was performed using styrene/dimethylbenzene (Chromabond) as solid phase and dichloromethane/acetone as liquid phase. GC-MS consisted of a TurboMass Gold (Perkin Helmer). Samples were injected (1 µL) in a 50 meter chromatographic column containing 5% phenyl 95% dimethylpolysiloxane with helium as carrier gas. The initial temperature of 50°C was increased to 320°C. The detection limit for pesticides in water was 0.05 µg/L.

Sixteen PAHs defined as prioritary by United States Environmental Protection Agency (US EPA), PCBs n° 28, 52, 101, 118, 138, 153, 180 and twenty five pesticides described in Sanchez et al. (2008b) were determined in sediment extracts using a multiresidue procedure. Sample preparation was performed on an ASE 300 system (Dionex). 10 g of sediments were mixed with 5 g of Celite and extracted using dichloromethane. The collected extracts were

evaporated using nitrogen flux and resuspended in methanol. After extract purification by Fluoresil, organic compounds were analysed using the same GC-MS method previously described. The detection limits in sediment were 50 mg/kg for pestides and 20 mg/kg for PCBs and PAHs.

2.3. Tissue handling

After capture, external examination was performed to identify and exclude fish exhibiting parasitism or morphological alterations. The fish were weighed, measured and immediately killed. Blood was collected, diluted 4-fold in a phosphate buffer (100 mM, pH 7.8) and stored in liquid nitrogen prior to VTG analysis. Liver, kidney and muscle were rapidly dissected, weighed and frozen in liquid nitrogen prior to homogenization and biochemical analysis. Gonads were removed, weighed and fixed in Boin to await histological analysis and gender determination.

For all fish, the condition factor (CF) was calculated according to Pottinger et al. (2002), while somatic indices for liver and gonad (HSI and GSI respectively) were calculated as (organ weight / fish weight)x100.

2.4. Biochemical and histological assays

All sampled fish were submitted to the same experimental protocol as fish from the Escrière river, except for CYP3A activity measurement. Livers were homogenized in an ice-cold phosphate buffer (100 mM, pH 7.8) with 20% glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a serine protease inhibitor. The homogenates were centrifuged at 10,000 g at 4°C, for 15 min and the post-mitochondrial fractions were used for biochemical assays. Total protein concentrations were determined using the method of Bradford (1976) with bovine serum albumin (Sigma-Aldrich Chemicals, France) as a standard. Hepatic biomarker assays, including EROD, GST, GPx and TBARS, were conducted, respectively, according to the methods of Flammarion et al. (1998a), Habig et al. (1974), Paglia and Valentine (1967) and

Ohkawa et al. (1979) adapted in microplate and optimized for stickleback as previously described (Sanchez et al., 2007). CYP3A activity was also measured in liver according to the method described by Miller et al. (2000), optimized for stickleback. Briefly, the post-mitochondrial fraction at 5 mg/L was added to 200 μM of 7-benzyloxyfluoromethylcoumarin and 2 μM of NADPH. The formation of 7-hydroxy-4-trifluoromethylcoumarin (HFC) is monitored by fluorescence using an excitation wavelength of 410 nm and an emission wavelength of 510 nm. Data was expressed as pmol of HFC formed/h/mg of proteins using HFC as standard.

As in the liver, all muscle samples were homogenized and centrifuged to obtain the post-mitochondrial fraction and total protein concentrations were determined. AChE activity was measured in muscle according to the method developed by Sturm et al. (2000). Butyril-cholinesterase was inhibited using tetraisopropyl pyrophosphoramide and the formation of 5-thio-2-nitro-benzoic acid, generated by the sample diluted at 0.5 g of proteins/L or by the standard of AChE from electric eel (Sigma-Aldrich Chemicals), was monitored at 405 nm. The results were expressed as nmol/min/mg of proteins.

VTG concentration was measured in total blood according to the method of Sanchez et al. (2005). This assay is based on a competition for the anti-VTG antibodies (GA-306, Biosense Laboratories, Bergen, Norway) diluted to 1:1,000 between standard-VTG coated on the wells of a microtitre plate at 25 ng/mL and free VTG in the sample or standard solutions.

Spiggin was quantified in kidney. For this, 200 μL of a strong denaturing buffer [100 mM Tris-HCl, 10 mM EDTA, 8 M urea, 2% SDS (w/v), and 200 mM β-mercaptoethanol; pH 8.5] were added to kidney and samples were heated at 70°C for 30 min. The enzyme-linked immunosorbent assay (ELISA) procedure for spiggin has been described in detail elsewhere (Sanchez et al., 2008c). This assay is based on a competition for the antibodies raised against a peptide sequence of spiggin (HRD-16) between protein derived from kidney extracts coated

on the wells of a microtitre plate and free spiggin in the sample. Spiggin contents were expressed as units/g of fish.

After fixation of gonads in Boin, the organs were dehydrated through a series of graded ethanol, cleared with toluene and embedded in paraffin. Sections were 7 µm thick, stained with haematoxylin-eosin-saffron, and observed under optical microscope.

2.5. Statistical analysis

All data are reported as mean ± standard deviation and the SPSS statistic 17.0 software was used for statistical analysis. Firstly, normal distribution and homoscedasticity of data were verified using Kolmogorov-Smirnov and Levene tests respectively (α=0.05). Since the data sets did not have a normal distribution and/or homogeny of variance, the biomarker data were log-transformed, using $F(x) = \log(1+x)$, prior to parametric analysis. Secondly, a two-way analysis of variance (ANOVA) was performed for each biomarker using sites and gender as factors. When sites by gender interaction was significant (α=0.05), male and female data were treated separately. Biomarker responses measured in fish from sites E2 and E3 were compared to biomarker responses recorded at site E1 using a t-test (α=0.05). Similarly, biomarker data from all Escrière sites were compared to "absolute" reference defined at the VDV site using a t-test (α=0.05).

3. Results

3.1. Contamination of Escrière sampling sites

Results of chemical analysis performed in water and sediment of Escriere sampling sites are presented in Table 1 and compared to concentrations measured in VDV reference sites. Except for Ni, Escrière sites were more contaminated by heavy metals that VDV reference site. Cd showed an increase of water concentration between upstream and downstream. For the other investigated metals, this trend was not observed and all Escrière sites were characterized by similar concentrations. Among the twenty five pesticides analysed, only

three of them were quantified in Escrière river: glyphosate, AMPA and metazachlore. Glyphosate was observed in all investigated sites with an increase of concentrations between upstream and downstream. AMPA was quantified only in sites E2 and E3, and metazachlore was observed only in site E1. In sediments, PAHs were detected with a sum of 16 prioritary compounds up to 15 mg/kg. PCBs were not detected.

3.2. Physiological indexes and biomarker responses in sticklebacks

For all investigated physiological indices (i.e. HSI, GSI and CF), no significant difference was recorded between data collected at the VDV reference site during a non-reproductive period and values measured in fish from the upstream E1 site (Table 2). This result indicates that fish from the Escrière river were not in breeding status, as confirmed by histological analysis of gonads. Indeed, male gonads were in an intermediate spermatogenesis with visible spermatozoa in the central part of the tubule only (Figure 2A). Gonads of female fish were characterized by a mix of previtellogenic oocytes and oocytes in the first stages of vitellogenesis that exhibit single small vacuoles in the cytoplasm (Figure 2B). No histological alteration such as ovotestis, fibrosis or necrosis, was observed at the investigated sites, but significant inductions of HSI and CF were recorded in fish from location E3, which could be due to the effects of water contamination.

For biochemical biomarkers, results are presented in Table 3 and show that reference selection is a critical point for data analysis and biomarker results interpretation. Indeed, there was no significant variation for EROD activity between the investigated Escrière locations (25.3±16.1, 31.7±20.2 and 27.8±17.4 pmol/min/mg prot. for E1, E2 and E3 respectively). However, EROD appeared as significantly induced when data from Escrière sites were compared to reference values previously established at VDV site (6.2±2.1 pmol/min/mg prot.). Similar results were obtained for AChE activity: there was no evidence of variation between Escrière locations, but a weak inhibition in fish from E1 (75+18 U/mg prot.) and E3

(77±16 U/mg prot.) was revealed when data were compared to AChE data from VDV sticklebacks (93±26 U/mg prot.). For GST data, too, a strong dependency on the reference site selection was identified. Compared to data from E1 (2,692±584 U/g prot.), E2 and E3 were characterized by low levels of GST (1,422±361 and 1,193±276 U/g prot. respectively) but if GST activities measured at the Escrière sites were compared to data from VDV (1,236±396 U/g prot.), E1 exhibited GST induction and no effect was recorded for E2 and E3.

In this study, no significant difference was observed for GPx activity, which appeared as the only gender-dependent parameter; the same can be concluded for TBARS, VTG and SPG levels between the upstream Escrière site and the VDV reference location. Moreover, no variation of variation of these biomarkers was recorded between all Escrière sites except for TBARS and VTG, which exhibited an induction in sticklebacks from the E3 site (123+52 nmol/g prot. and 9.4+7.2 μg/L respectively).

A significant decrease of CYP3A activity was recorded in fish from the E3 site (4.8±1.6 pmol/h/mg prot.) compared to E1 (7.5±2.3 pmol/h/mg prot.), but, because of a lack of data on the CYP3A basal level in stickleback, it is not possible to compare this result with other reference systems.

4. Discussion

The aim of this work was to evaluate advantages and limits of two reference systems for biomarker data analysis. Indeed, assessment of ecological status of water bodies in the WFD context is based on the extent of deviation from previously established reference conditions. Hence, the application of this concept cannot be ignored when introducing biomarkers in monitoring regimes established under the WFD (Sanchez et al., 2009). The results of the present study show that reference selection is a crucial point for a better interpretation of biomarker response profiles measured in wild fish. For example, EROD activity is considered

as a well described biomarker in fish to reflect exposure to organic pollutants such as PAHs, PCBs and other dioxin-like compounds (Whyte et al., 2000). Fish from the Escrière river were characterized by a lack of EROD variation between investigated sites (Table 2). Hence, it could be easy to conclude that sticklebacks from locations E2 and E3 were not exposed to an EROD inducer. But comparison of EROD data from the Escrière river and reference data previously established for the stickleback (Sanchez et al., 2008b) reveals EROD induction in fish sampled at all Escrière locations. On the basis of extensive knowledge of EROD activity in fish including the stickleback, it is possible to compare data recorded in the present work with other field and laboratory data. It appears that EROD activity measured in fish from the VDV site is similar to activity values reported in other low- contamination sites (Sanchez et al., 2007, 2008a) and in control fish from laboratory experiments (Holm et al., 1993, 1994; Andersson et al., 2007; Sanchez et al., 2008d). In these conditions, EROD activity can be considered as induced in fish collected at all Escrière sampling sites. This result is in accordance with the concentrations of 16 priority PAHs measured in sediments from the Escrière river that were up to 15 mg/kg (Table 1).

This study provides similar examples for other known biomarkers such as GST and AChE (Table 3). Significant differences were recorded between responses of fish from VDV and the E1 reference site, but values observed for VDV fish were in accordance with activities of both biomarkers measured in other low-contamination sites (Sanchez et al., 2007; Sanchez et al., 2008a; Sturm et al., 1999).Moreover, comparison with data of fish from Escrière sampling sites reveals induction of GST at E1 site and inhibition of AChE at E1 and E3 sites. These results could be linked to the presence, in Escrière river, of pesticides such as glyphosate, a documented AChE inhibitor (Glusczak et al., 2006) or other organophosphorous pesticides not investigated in the present work. These results support also the use of "absolute" references to correctly interpret biomarker response profiles, using knowledge of the

physiological level of investigated parameters. Selection of an "absolute" reference is, however, pragmatic, due to high contamination of aquatic ecosystems as shown by chemical analyses performed in sediments from the VDV site, which revealed the occurrence of PAHs, estradiol and alkylphenols (Kinani et al., 2010). Moreover, biotic and abiotic factors can also alter biomarker base levels (Hansson et al., 2006). Also, the selection of an "absolute" reference requires a rigorous process. In a case study investigating the responses of wild chubs to environmental contamination across the southern Belgium hydrological network, Mayon et al. (2006) selected reference sites using the procedure dictated by the European Water Framework Directive to reduce anthropogenic impacts. In this procedure, hydrological regime, river connectivity, morphological conditions, toxic acidification and nutrient organic inputs are considered and only sites ranked as no or low impacted sites are considered as reference sites. To complete this procedure and to decrease variability of measured responses, the authors worked at sampling sites located within the same hydro-ecoregion and on a low range of Huet' zonation. In the present work, similar criteria were considered.The VDV reference site is located in the same hydro-ecoregion as the Escrière river (i.e. Tables calcaires; Wasson et al., 2002) and has been selected for its lack of chemical pollutant sources and the lack of fish assemblage disturbance (Sanchez et al., 2008b). To complete this selection process, data used for biomarker result interpretation were collected in the same season, in fish exhibiting a similar physiological status as shown by somatic index measurements (Table 2) and gonad histology (Figure 2). Indeed, histological analysis of gonads revealed that male and female sticklebacks from the Escrière river were in intermediate spermatogenesis and at the beginning of vitellogenesis, respectively. This status is consistent with the fact that sampled fish were not in a reproductive status while these stages of gonad development are encountered in wild stickleback populations, during autumn, after the end of the spawning period (Sokolowska and Kulczykowska, 2006). In these

conditions, it appears easy to discuss biomarker responses of several biomarkers such as endocrine disruption parameters (i.e. VTG and SPG) that were characterized by a lack of base level in both reference systems (Table 3).

The strategy based on an "absolute" reference has important limitations when analysis responses for novel biomarkers. To our knowledge, this study is the first application of CYP3A activity as a biomarker in the stickleback and no data are available to determine a valuable "absolute" reference. CYP3A activity is described as a powerful end-point to investigate biotransformation of emerging pollutants including pharmaceuticals (Hasselberg et al., 2008; Hegelund et al., 2004) but it is scarcely investigated in field studies to assess exposure of wild populations to these pollutants (McArdle et al., 2004; Gagné et al., 2008). In this study, the CYP3A response profile exhibited inter-site differences while fish from E3 were characterized by low activity compared to other investigated Escrière sites. However, due to a lack of knowledge of this biomarker in the stickleback, it is not possible to determine whether CYP3A activity was induced in fish from E1 and E2 sites or whether it was inhibited in fish living at the E3 downstream site. Recently, Kinani et al. (2010) reported a large contamination of sediments from rivers in the north of France by human PXR activating substances and the absence of anti-PXR activity in these samples, which could argue for CYP3A activity induction. Further studies are, however, needed to characterize a base level and induced responses of CYP3A in the stickleback and to analyse accurately CYP3A responses measured in wild populations.

5. Conclusion

This study was designed to evaluate the ecotoxicological effects of a small city located in an agricultural water basin, on wild sticklebacks living in an adjacent river. This study offers a valuable opportunity to compare advantages and limits of two reference systems, i.e., the "absolute" and the relative reference, for biomarker analysis in an environmental

biomonitoring context. For this purpose, a set of biochemical biomarkers was measured in adult sticklebacks collected in an urban and agricultural water basin. Biological responses measured in both downstream sampled sites were compared with responses collected in the upstream site and with biomarker base levels previously established for stickleback living in the north of France. It appears clearly that the investigated city disturbed CYP3A activity but also induced hepatic lipoperoxidation and vitellogenin. Our results show that reference selection is a major critical factor for accurate interpretation of biomarker response profiles measured in environmental biomonitoring using wild fish sampling. Indeed, application of a relative reference showed that the investigated city has a moderate effect on the physiological status of fish from the Escrière river. Application of an "absolute" reference, however, showed a cumulative effect of contamination, while the observed impacts on the Escriere river upstream of the city enabled the effects induced by the urban area to be clearly identified. Due to the constraints on reference selection, both reference systems appear as pragmatic and complementary. The use of various references associated with good knowledge of investigated biomarkers in selected fish species appears as the best way to reduce the risk of errors in biomarker response analysis. Hence, further studies are needed to characterize the effects of biotic and abiotic confounding factors on biomarker base levels and to define natural variability ranges of biomarkers in a large geographical scale. The results of this research will contribute to the introduction of biomarker measurements in regular monitoring programmes. .

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Table 1. Results of chemical analysis performed in water and sediment of Escrière river. na : not available and nd: not detected.

	VDV[1]	E1	E2	E3
Cd (water) µg/L	nd	0.08	1.3	1.4
Cr (water) µg/L	0.7	nd	nd	nd
Cu (water) µg/L	0.7	1.4	1.8	1.6
Ni (water) µg/L	4.0	3.8	4.1	3.9
Pb (water) µg/L	0.3	8.4	9.2	9.0
Zn (water) µg/L	7.0	10.1	10.0	11.2
Glyphosate (water) µg/L	na	0.6	1.2	1.4
AMPA (water) µg/L	na	nd	0.8	1.0
Metazochlore (water) µg/L	nd	0.4	nd	nd
Sum of 16 PAH (sediment) mg/kg	0.74	15.0	17.3	16.9

[1] Data from Sanchez et al. (2008b).

Table 2. Morphological parameters for three-spined sticklebacks caught at the different sampling sites. Escriere sites annoted with the same letter are not statiscically different ($p<0.05$). * represents a significant difference withe the VDV site ($p<0.05$).

	Gender	**VDV**	**E1**	**E2**	**E3**
HSI %	M and F	2.5±0.6	2.5±0.7[a]	2.7±1.0[a]	4.3±1.4[b]*
GSI %	M and F	1.3±0.9	2.3±2.9[a]	2.0±1.0[a]	2.1±1.7[a]
CF	M and F	0.8±0.1	0.7±0.1[a]	0.8±0.1[ab]	0.9±0.1[b]

Table 3. Biomarker response for three-spined stickleback caught at the different sampling sites. Escriere sites annoted with the same letter are not statiscically different ($p<0.05$). * represents a significant difference with the VDV site ($p<0.05$). na : not available and nd: not detected.

	Gender	**VDV**	**E1**	**E2**	**E3**
EROD pmol/min/mg	M and F	6.2±2.1	25.3±16.1[a]*	31.7±20.2[a]*	27.8±17.4[a]*
CYP3A pmol/h/mg	M and F	na	7.5±2.3[a]	9.1±2.9[a]	4.8±1.6[b]
GST U/g prot.	M and F	1,236±396	2692±584[a]*	1422±361[b]	1193±276[b]
GPx U/g prot.	M F	95±35 135±41	88±30[a] 151±62[a]	104±47[a] 148±50[a]	99±33[a] 127±43[a]
TBARS nmol/g prot.	M and F	49.5±22	71.8+30.2[a]	89.6+36.1[ab]*	123+52[b]*
AChE U/mg prot.	M and F	93±26	75+18[a]*	84+21[a]	77+16[a]*
VTG µg/L	M	nd	nd	nd	9.4+7.2
SPG U/g fish	F	nd	nd	nd	nd

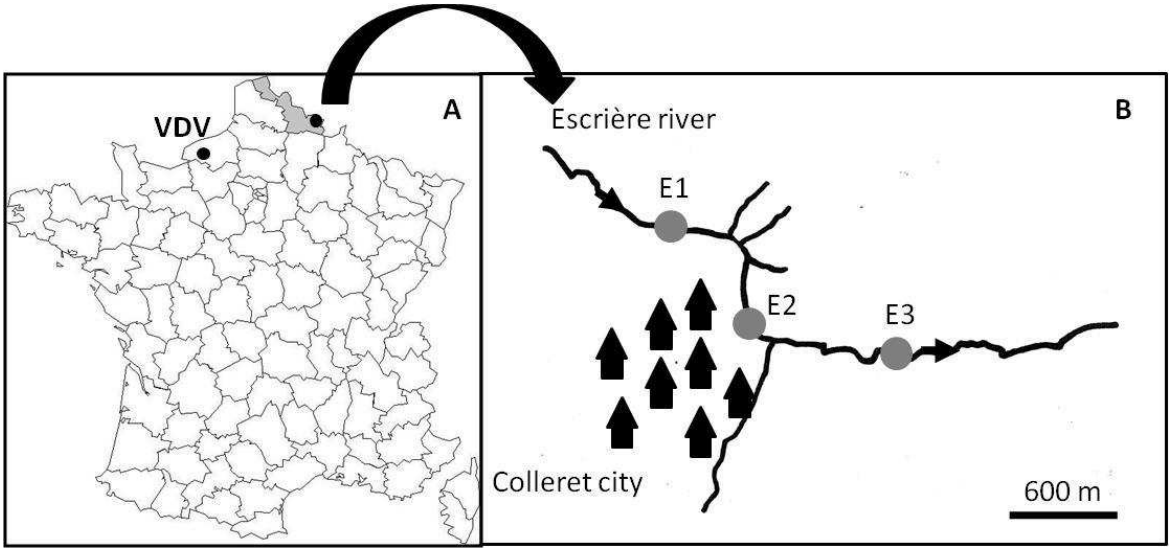

Figure 1 : Location of Escrière river sampling sites. A represents location of Escrière river and VDV reference site in France. B represents location of selected sampling sites in Escrière river basin.

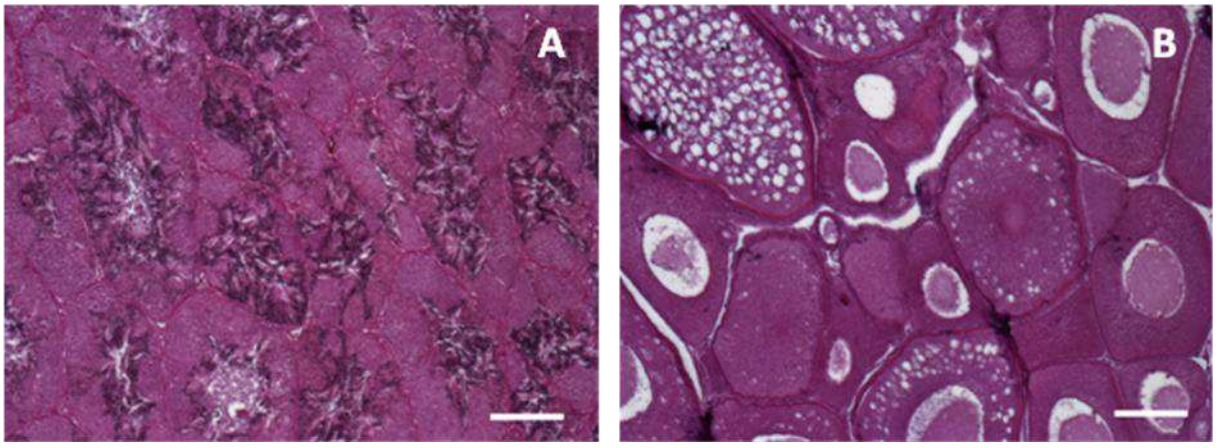

Figure 2 : Histological observation of gonad development stage of male (A) and female (B) sticklebacks. 7 µm sections were stained with haematoxylin-eosin-saffron and observed under optical microscope. White bars represent 100 µm.