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Abstract

A comprehensive evaluation of organic contamination was performed in sediments sampled in two reference and three impacted small streams where endocrine disruptive (ED) effects in fish have been evidenced. The approach combined quantitative chemical analyses of more than 50 ED chemicals and *in vitro* bioassays allowing the quantification of receptor-mediated activities, namely estrogen (ER), androgen (AR), dioxin (AhR) and pregnane X (PXR) receptors. At the most impacted sites, chemical analyses showed the presence of natural estrogens, organochlorine pesticides, parabens, polycyclic aromatic hydrocarbons (16 PAHs), bisphenol A and alkylphenols, while synthetic steroids, myco-estrogens and phyto-estrogens were not detected. Determination of toxic-equivalent amounts showed that 28 to 96% of estrogenic activities in bioassays (0.2-6.3 ng/g 17β-estradiol equivalents) were explained by 17β-estradiol and estrone. PAHs were major contributors (20-60%) to the total dioxin-like activities. Interestingly, high PXR and (anti)AR activities were detected; however, analysed compounds could not explain the measured biological activities.

Keywords: river sediment; endocrine disrupting chemicals; in vitro bioassays; GC-MS & LC-MS analysis; mass balance analysis

Capsule : Multiple endocrine disrupting chemicals (ER, AR, AhR and PXR ligands) are detected in French river sediments using a panel of *in vitro* bioassays and analytical methods.

1. Introduction

The rapid industrial and urban development which occurred in the second half of the 20th century allowed the emergence of millions of persistent anthropogenic chemicals in our environment without prior study of their toxicity. In recent years, a newly defined category of these chemicals, with the potential to interact with the endocrine system have emerged and aroused considerable interest of scientific communities. Named endocrine disrupting-chemicals (EDCs), these substances are defined as "exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones which are responsible for maintenance of homeostasis, reproduction, development and/or behavior" (Kavlock et al., 1996). In the aquatic environment, occurring EDCs are very diverse in terms of chemical nature and origins (Vos et al., 2000).

Up to date, most attention has been directed to identifying estrogenic chemicals, i.e. those capable of binding to and activating the estrogen receptor (ER), because many of the effects reported in wildlife appear to be a consequence of feminization of males (Sumpter et al., 2005). However, many environmental contaminants can interfere with other molecular targets involved in the regulation of the endocrine system. These include other nuclear receptors like androgen receptor (AR), pregnane X receptor (PXR, e.g. Mnif et al., 2007) as well as steroidogenesis enzymes like aromatase (e.g. Laville et al., 2006). EDCs can also act via indirect mechanisms such as (anti)estrogenic effect mediated by aryl hydrocarbon receptor (AhR) ligands through an ER/AhR cross-talk dependent mechanism (Othake et al., 2003, 2007). Therefore, a comprehensive assessment of EDC hazard should take into account the ability of chemicals to interfere with different targets of the endocrine system (Houtman et al., 2004).

Regarding the large diversity of EDCs and their effects, bioanalytical approaches using mechanism-based biological screening tools have emerged to monitor such substances in

complex environmental mixtures (Eggen and Segner, 2003). In particular, *in vitro* assays using reporter gene activation in stably transfected cell lines provide robust, sensitive and specific bioassays to screen and quantify endocrine activities in environmental samples. Combining such tools with powerful chemical analyses within integrated approaches permits to identify key toxicants to be monitored in the environment (Brack, 2003). By using such bioanalytical approaches, the occurrence of EDCs and their effects to aquatic organisms has been several times reported in different industrialised countries like the United Kingdom (e.g. Jobling et al., 2006) or the Netherlands (e.g. Vethaak et al., 2005). However in France, much fewer data have been reported. Recent studies showed occurrence of steroid estrogens and alkylphenols in effluents from sewage treatment plants (Labadie and Budzinski, 2005, Muller et al., 2008) or in water and sediment from the Seine River (Fenet et al., 2003; Cargouet et al., 2004). More recently, abnormal elevated levels of vitellogenin and spigging, used as biomarkers of estrogen and androgen exposure respectively, have been reported in three-spine stickleback (*Gasterosteus aculeatus*) sampled in different small streams subjected to diffuse pollution like agricultural run-off or domestic effluents (Sanchez et al., 2008). Thus, more investigation is needed in order to characterise and identify key EDCs in French aquatic ecosystems.

This study aimed at performing a comprehensive evaluation of the chemical contamination by EDCs in sediments sampled in small streams at sites where endocrine disruptive effects in fish have been previously evidenced (Sanchez et al., 2007, Sanchez et al., 2008). The five selected sites, located in the North of France, were representative of different levels of water and ecological quality (Table 1). For this purpose, we used a combined approach involving i) targeted chemical analyses of more than 50 chemicals (Table 2) selected on the basis of both their environmental occurrence and their known EDC potency, and ii) a panel of *in vitro* bioassays that allowed the detection of different receptor-mediated activities, namely ER, AR,

AhR and PXR. Since all activities were detected in at least two of the five studied sites, the contribution of analyzed EDCs in the biological activities detected by the bioassays was estimated by comparing toxic-equivalent quantities from both approaches.

2. Materials and methods

2.1. Chemicals and reagents

List, abbreviation and source of analytical standards are given in Table 2. Flutamide (Flut), 3-(4,5-dimethylthiazol-2-ol)-2,5-diphenyltetrasodium bromide tetrazolium (MTT), 5α-dihydrotestosterone (DHT), as well as the analytical internal standards including [$^{13}C_6$]4-n-nonylphenol, [$^{2}H_2$]17β-estradiol, [$^{2}H_{10}$]phenanthrene, [$^{2}H_{10}$]acenaphthene, [$^{2}H_{12}$]perylene and [$^{2}H_{12}$]chrysene were purchased from Sigma-Aldrich (St Quentin Fallavier, France). ICI 182,780 (ICI) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were obtained from Tocris Bioscience (Ellisville, USA) and Promochem (Molsheim, France), respectively. All standards were of 98.1–99.9% purity. Chromatographic-grade solvents: acetone, methanol, acetonitrile, dimethylsulfoxide (DMSO) and n-hexane were also purchased from Sigma-Aldrich. Stock solutions of β-E2 2.72 g/L, DHT 2.90 g/L and B[a]P 2.52 g/L were prepared by dissolving crystalline compound in DMSO. A solution of TCDD was prepared by drying an aliquot of TCDD in methanol under a nitrogen stream and dissolving the residue in DMSO to reach a concentration of 3.22 g/L.

2.2. Site description and sampling

Surface sediment samples were collected in five rivers located in the North of France during July 2004. The selected stations presented different levels of impacts in terms of anthropogenic pressures, chemical contamination and impacts on fish populations (Table 1):

the Aisne (Ais) and the Vallon du Vivier (VdV) rivers were considered as reference sites; the Lézarde (Lez) as a moderately impacted site and the Réveillon (Rév) and Rhonelle (Rho) as heavily impacted sites. At each site, at least five grab sediment samples were taken alongside the river section of the study sites and were pooled to obtain an average sediment sample and to minimize intra-site variability. All sediment samples were passed through a 2 mm sieve pore, stored in aluminium boxes and immediately transferred to the laboratory where they were stored at -20 °C in order to inhibit biological activity until extraction.

2.3. Extraction procedure

The sediment samples were extracted as previously described (Kinani et al., 2008a). Briefly, 5 g of lyophilised and homogenised sediment were extracted three times with 10 mL of a hexane/acetone (2:1, v/v) mixture. For each extraction step, the sample was sonicated 10 min (ENMA Transsonic 460/H, Frequency 50-60 Hz, Germany) at room temperature. The extracts were combined and the supernatant was transferred to a 50 mL vial containing 1.0 g of anhydrous sodium sulphate. The organic extracts were then centrifuged for 10 min at 3000 rpm and the supernatant was collected, filtered onto a 0.45 µm Acrodisc CRPTFE Syringe filter (GelmanSciences, USA), reduced to about 1 mL using rotary evaporation at 30 °C or lower, evaporated to complete dryness under a gentle nitrogen stream and reconstituted into 1 mL of the extraction solvent. The final extract was then divided into two parts, the first one for chemical analysis and the second one for bioassay testing. The extraction solvent was subsequently replaced by DMSO for bioassay experiments.

2.4. Cell cultures and in vitro bioassays

2.4.1. Estrogenic, PXR and (anti-)androgenic activities in reporter gene assays

136 The estrogenic, PXR and (anti-)androgenic activities of the extracts were monitored by using
137 the MELN, HG_5LN-PXR and MDA-kb2 reporter cell lines, respectively. The MELN cell line
138 was obtained by stable transfection of MCF-7 human breast cancer cells by an ERE-βGlob-
139 Luc-SVNeo plasmid (Balaguer et al., 2001). The HG_5LN-PXR cell line was derived from
140 HeLa cells that were first stably transfected with GAL4RE_5-bGlob-Luc-SV-Neo (HG_5LN
141 cells) before being stably transfected with the pSG5-GAL4(DBD)-hPXR(LBD)-puro plasmid
142 (Lemaire et al., 2006). The MDA-kb2 cell line (ATCC, #CRL-2713) was derived from the
143 MDA-MB-453 human breast cancer cells. They were stably transfected by a MMTV
144 promoter-luciferase plasmid construct, which is under the control of endogenous AR and
145 glucocorticoid receptor (GR) (Wilson et al., 2002). All reporter cell lines were routinely
146 cultured in phenol red containing Dulbecco's Modified Eagle's Medium (DMEM),
147 supplemented with 5% foetal calf serum (FCS), 1% nonessential amino acids and
148 penicillin/streptomycin (50 U/mL each) in a 5% CO_2 humidified atmosphere at 37 °C. For
149 experiments, cells were left to incubate for two days in phenol red free DMEM supplemented
150 with 3% dextran charcoal coated-FCS (DCC medium) before seeded in white opaque 96-
151 wells culture plates at a density of 50 000 cells per well. Serial dilutions of reference
152 chemicals or organic extracts were added in triplicates 24 h later and then left to incubate for
153 16 h. In all assays, DMSO in the culture medium was always at 0.5% v/v, including in cell
154 controls. At this concentration, it did not affect cell viability or luciferase activity. At the end
155 of the incubation period, the medium was removed and replaced by 50 µL of DCC medium
156 containing 0.3 mM of D-luciferin and the luminescence signal was measured in living cells
157 for 2 seconds per well with a microtiter plate luminometer (µBeta, Wallac). Relative
158 luminescence units (RLU) were converted to relative response units expressed as percent of
159 maximal luciferase activity induced by the positive controls: E2 10 nM, SR12813 0.3 µM,
160 DHT 10 nM (for AR agonist activity) and DHT 0.3 nM (for AR antagonist activity) in

MELN, HG_5LN-PXR and MDA-kb2 cell lines, respectively. For ER and AR agonistic responses, the specificity of luciferase induction by samples was checked by co-exposure experiments with 0.1 µM ICI182,780 or 1 µM flutamide, as reference ER and AR antagonists, respectively.

2.4.2. Dioxin-like activity in PLHC-1 cell line

The fish hepatic PLHC-1 cell line (ATCC, #CRL-2406) was described by Ryan and Hightower (1994). The cells were routinely grown at 30 °C in E-MEM culture media supplemented with 10 % FCS and 1 % antibiotics in a 5 % CO_2 humidified atmosphere. For experiments, cells were seeded in 96-well plates at a density of approximately 50 000 cells per well. After 24 hours of incubation, cells were exposed to test chemicals or sample extracts for either a 4 h or 24 h incubation period, in order to differentiate between dioxin-like compounds that are rapidly metabolized (e.g. PAHs) in the cells and dioxin-like chemicals that are persistent in the cells (e.g. dioxins) (Louiz et al., 2008). Then, plates were processed for 7-ethoxyresorufin-O-deethylase (EROD) activity in intact cells, following the protocol previously described by Laville et al. (2004). Results were expressed as percent of EROD activity induced by the positive control (TCDD 1 nM).

2.4.3. Cell viability

The effect of test compounds or samples on cellular viability in the different cell lines was evaluated by using the methyl-thiazol-tetrazolium (MTT) assay (Mosmann, 1983), as previously described (Laville et al., 2004).

2.5. Chemical analyses

Two mass spectrometers coupled with chromatography have been used for the quantitative analysis: a “CP3800” gas chromatograph system equipped with a “CP8400” autosampler and coupled to a “Saturn 2000” ion trap mass spectrometer (Varian, Les Ulis, France) for the GC-

MS and a “alliance 2690” liquid chromatography coupled to a Q-TOF Premier (Waters, France) mass spectrometer for LC-MS/MS. Before performing the chemical analysis, a portion of each extract was purified according to a method previously described by Hartmann et al., (2007).

2.5.1. GC-MS and GC-MS/MS analyses

The chromatographic separation was performed with a 60 m “Factor four VF-10-MS” (10% phenyl, 90 % methylpolysiloxane) capillary column (internal diameter: 0.25 mm, film thickness: 0.25 µm) from Varian. All experiments were performed by automatically injecting 2.0 µL of sample in the splitless mode at a rate of 50 µL/s. Helium (99.999% purity) was used as the carrier gas at a constant flow of 1.0 mL/min for PAHs analysis and at 1.4 mL/min for the other target compounds; and was held a constant flow by electronic pressure control. The injector temperature was set to300 °C. The split valve opened after 1 min, with a split ratio of 35/100. The manifold, ion trap source and transfer line temperatures were set to 120, 220 and 300 °C, respectively.

For the GC-MS analysis, except for organochlorines pesticides, all acquisition methods have been previously described, the corresponding references being reported on Table 2 according to the investigated chemical families. For HCB, γ-HCH, vinclozolin, metolachlor, α-endosulfan, *o,p’*-DDT, β-endosulfan, methoxychlor and fenarimol analysis, the capillary column was initially set at 80°C for thirty seconds, then ramped up to 280°C at 15 °C/min. After 4.0 min at this value, the temperature was finally increased at 20 °C/min up to 350, where it was held for 3.67 min. The total duration of GC analysis was 21 min. Ions were formed under electro-ionization at 70 eV with an emission current of 80 µA. The mass spectrometer was operated using a hybrid acquisition mode alternating MS/MS and SIS detection.

2.5.2. LC-MS/MS analyses

LC/MS is equipped with an electrospray ionization (ESI) source operated in negative-ion mode. The analytical column used was a Pursuit C18 (150 mm x 2.1 mm I.D., 5µm particle size, Varian, France) and a mobile phase consisting of acetonitrile (A) and water (B). The gradient conditions were as followed: 25% of (A) and 75% of (B) for 2 min, followed by a linear increase to 75% (A) at 16 min and 2 min hold at 75% (A). The flow rate was 0.2 mL/min and the injection volume was 20 µL. Only phytoestrogens were analysed by LC-MS. The mass spectrometer was operated in order to record the MS/MS spectrum of deprotonated pseudomolecular ion $[M-H]^-$. Collisions energies have been optimized between 20 to 30 eV for each compound to obtain three characteristic ions. The optimized instrument conditions were as followed: capillary voltage -3.4 kV, cone voltage, 50 V; multiplier voltage, 2250 V; source temperature, 100°C; desolvatation temperature, 450°C. Nitrogen was used as both the nebulizing (50 L/h) and desolvatation gas (700 L/h). Argon was used as collision gas at flow 0.28 (mL/min).

2.6. Data analysis and determination of bioassay- and instrumental-derived toxic-equivalents

Sigmoid dose-response curves and efficient concentrations (i.e. EC_{25} and EC_{50}, corresponding to concentrations of sediment extracts and chemical standards giving respectively 25 and 50% of the maximum luciferase or EROD activities) were determined with the Regtox 7.5 Microsoft Excel™ macro by using Hill equation (Vindimian et al., 1983) and freely available at http://eric.vindimian.9online.fr. In all bioassays, significant responses were defined as those greater than two times the standard deviation of the response obtained with DMSO (solvent control). The dioxin-like, estrogenic, (anti)androgenic and PXR activities in samples derived from bioassays were expressed as BaP- or TCDD-, E2-, DHT-, Flu- and SR12813-equivalents (Bio-ref-EQs), respectively, which were determined as the ratio of the EC_{25} of the reference

chemicals expressed as ng/L or µg/L to that of the sample expressed as equivalent gram of dry weight sediment per litre (g EQ/L).

In order to estimate the contribution of analysed compounds to the total activity detected by a bioassay, the measured concentrations (as ng/g sed wt) were converted to toxic-equivalent activities derived from chemical analysis (Chem-ref-EQs), that is Chem-BaP-EQ, Chem-TCDD-EQ, Chem-E2-EQ, Chem-DHT-EQ, Chem-Flu-EQ and Chem-SR12813-EQ, in each respective bioassay. The Chem-ref-EQ were calculated according to the following equation: Chem-ref-EQ = $\sum (C_i \times \text{ref-}EF_i)$, where, for a given chemical *i*, C_i is the measured concentration in a sample and ref-EF_i is the inducing/inhibiting equivalent factor relative to the reference ligand (i.e. BaP, TCDD, E2, DHT, Flu or SR12813) in a given bioassay. The equivalent factors were calculated as follows: ref-EF_i = EC_{25} of reference compound / EC_{25} of test compound *i*, on mass basis. Unless otherwise specified in Tables 3-5, ref-EFs of each analysed compound were determined experimentally in each bioassay by establishing dose-response curves for individual standard chemicals and by comparing them to that of the reference compound. For instance, the 21 individual molecules listed in Table 4 were tested for their ability to induce luciferase activity in MELN cells in our assay condition, and their E2-equivalent factors (EEF) were then determined and used to calculate Chem-E2-EQ in the samples. Finally, the ratio Chem-ref-EQ/Bio-ref-EQ allowed evaluating the contribution of quantified compounds to the biological activity detected by the bioassay, i.e. a ratio near 100% means that all analysed compounds were explicative for biological results; otherwise other non analysed compounds may be present in the samples.

3. Results

3.1. Dioxin-like activity in river sediment extracts

The dose-response curves for EROD induction by all organic extracts are shown in Figure 1. All samples elicited significant EROD activity, indicating a general contamination by dioxin-like compounds in all studied sites. Sample responses varied greatly depending on the studied site and exposure duration. Except for the Ais site after 24 h of exposure, the dose-response curves were fairly complete and almost parallel to the dose-response curves for reference chemicals. This allowed us to use EC_{50} values for calculation of biological BaP-EQs and TCCD-EQs (summarized in Table 3). The highest values were found for Lez site followed by Rev, Rho, VdV and Ais. For all sediment extracts, EROD induction potency based on sample concentration was higher after 4 h (Figure 1a) than after 24 h of exposure (Figure 1b) by approximately one to three orders. This indicates a major contribution of non persistent compounds, like PAHs, in the observed dioxin-like activities.

In order to test this hypothesis, the 16 priority PAHs were analysed in the extracts (Table 3). Total PAHs concentrations indicate large between-site variations ranging from low contamination levels in Ais site (0.22 µg/g d.w.) up to very high levels in Rev (11.98 µg/g d.w.) and Lez (23.08 µg/g) d.w. sites. All sediment samples were dominated by high molecular mass PAHs (4- to 6-ring).

The results given in Figure 2 showed that chemical- and bioassay-derived BaP-EQs and TCCD-EQs were highly correlated (r^2= 0.99 and 0.97, respectively), which confirmed the involvement of PAHs in the detected biological activities at both exposure durations. However, in all sediment extracts, the Chem-EQs were significantly lower than the Bio-EQs, indicating that only a part of activity was explained by analyzed PAHs, which accounted for approximately 20 to 60 % and 16 to 40 % of the BaP-EQs and TCCD-EQs measured in PLHC-1, respectively.

3.2. Estrogenic activity in river sediment extracts

285 The presence of estrogenic compounds in sediment extracts was tested in the MELN reporter
286 cell line (Figure 3). Significant dose-dependent induction of luciferase activity was obtained
287 for all tested sediment extracts; the response magnitudes varied between 34 % and 67 % of
288 the maximal response elicited by β-E2. Moreover, co-exposure with the pure anti-estrogen
289 ICI-182,780 led to inhibition of the luminescent signal (data not shown), thus showing the
290 specific involvement of the ER in the detected effects and indicating that estrogenic
291 compounds were present in samples. Because the slopes of dose-response curves for different
292 sediment extracts were not parallel to that of β-E2, the use of EC_{25} was chosen to derive Bio-
293 E2-EQs. The Bio-E2-EQs values in the different extracts of sediment samples ranged from
294 0.20 to 6.43 ng/g d.w (Table 4), with the highest activity in the Réveillon sediment extract.
295 Overall, the results presented in Table 4 showed that E1, β-E2, 1OHPyr and BPA were
296 detected in all samples, while the synthetic estrogens were never detected. The Réveillon site
297 was the most contaminated by xeno-estrogens, with measurable levels of natural estrogens,
298 alkylphenols, PAH metabolites, parabens, chlorinated pesticides, as well as detectable levels
299 of the zearalenone metabolites α-ZAL and β-ZAL. α-E2 was detected at Rhonelle and Aisne
300 sites; equol was present at Rhonelle site, while other myco- and phyto-estrogens were not
301 detected.
302 In Aisne, Vallon du Vivier and Lézarde sediments, the Bio-E2-EQs were totally explained
303 only by the presence of E1 and β-E2. However, differences between chemical and biological
304 measurements were observed for Réveillon and Rhonelle sediments with Chem-E2-EQs /
305 Biol-E2-EQs ratio values of 28 and 35%, respectively. This suggests that these sediments may
306 contain estrogenic substances that were not included in our analytical methods.
307
308 ***3.3. (Anti) androgenic activity in river sediment extracts***

309 The dose-response curves show detectable androgenic activity in Aisne and Rhonelle samples
310 (Figure 4a), giving bioassay derived Bio-DHT-EQs values of 0.40 and 3.60 ng/g d.w.,
311 respectively. No androgenic activity was observed in the total sediment extract of Vallon du
312 Vivier, Lézarde and Réveillon sediments. In DHT co-exposure experiments (Figure 4b),
313 significant antiandrogenic activities were detected in Vallon du Vivier, Lézarde and Réveillon
314 samples, with respective Flu-EQs of 1.1, 7.4 and 32.5 µg/g d.w. By using the MTT test, no
315 cytotoxic effects could be observed for any working concentration of chemicals or organic
316 extracts (data not shown), thus indicating a specific effect on luciferase expression.
317 Determination of Chem-Flu-EQs showed that several anti-androgenic compounds were
318 present, but at concentrations that could explain only a very minor part of the observed
319 biological anti-androgenic activities (Table 5).
320
321 ***3.4. Detection of PXR ligands in river sediment extracts***
322 No anti-PXR activity was noted in these samples (data not shown). However, all five samples
323 induced PXR activation in a dose-response manner (Figure 5a). The response magnitudes
324 were always inferior to the maximum response produced by positive control (SR 12813 at 3
325 µM), and ranged from 30 % to 73 %. Nevertheless, luciferase induction was specific to PXR
326 activation since no luciferase increase was noted in the parental HG_5LN cell line, which
327 expresses only the GAL4-luciferase construct (Figure 5b). In HG_5LN cell line, inhibition of
328 luciferase at high concentrations of Rev and Lez extracts was noted and reflected early toxic
329 events.
330 The low Chem-SR12813-EQ/Bio-SR12813 ratios showed that 4tOP, 4nNP, βE2, E1, BPA,
331 endosulfan and *o,p'*-DDT, which are known PXR ligands, poorly contributed to Bio-
332 SR12813-EQs in the samples (Table 5). Thus, the detected PXR activities were due to other
333 non analysed compounds.

4. Discussion

In vitro profiling of sediment extracts showed multiple contaminations by dioxin-like and endocrine active chemicals in small streams subjected to diffuse anthropogenic pollution. The natural estrogens βE2 and E1 and of PAH-like compounds were identified as main contributors to estrogenic and dioxin-like activities, respectively, as determined by the bioassays. Conversely, (anti)androgenic and PXR-mediated activities were detected but the responsible compounds could not be identified using targeted chemical analyses.

4.1. PAHs and EROD inducing compounds

To some extent, the levels of PAHs measured in this study were in line with previous studies in French freshwater watersheds. For instance, total PAH content ranged from 1.2 to 12.5 µg/g of dry sediment at various sites from the Seine estuary (Cachot et al., 2006), and from 2.3 to 41.3 µg/g in the Seine and Marne rivers sampled at urban sites nearby Paris (Ollivon et al., 2002). In our study, the highest PAH levels were detected in areas subjected to mixed anthropogenic pressures like the Réveillon, located in urban area upstream of Paris, and the Lézarde river, located nearby a highway road. Conversely, the Aisne site was located in a non urbanized area and was logically found as low contaminated by PAHs.

In all samples, PAH-like compounds were major contributors of dioxin-like activity, which mostly explained by PAHs with molecular weights (M.W.) of 228 and 252; however analyzed PAHs explained only part of overall activity in PLHC-1 cells. This is not surprising since the priority PAHs are used as qualitative tracers of pollution and we noted that other high molecular weight PAHs, presumably active, were present in the samples (Figure 2). Indeed, fullscan chromatogram of Lézarde extract (data not shown) showed the presence of detectable concentrations of several isomers of Chry (M.W. 228) and BaP (M.W. 252) that probably

359 accounted for the detected activity in PLHC-1 cells, although this could not be tested in this
360 study. Similar conclusions have been reported by other studies that showed that a major
361 portion of AhR activity in river sediments was caused by nonpriority PAHs such as
362 methylated PAHs (e.g. Hollert et al., 2002; Brack et al., 2005). In addition, many other
363 dioxin-like compounds such as polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated
364 dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), polychlorinated naphthalenes
365 (PCNs) and some organochlorine pesticides, which were not included in the analyses, might
366 also explain the differences between chemical and biological analyses.

367 ***4.2. (Xeno) Estrogens***

368 The contamination of aquatic ecosystems by estrogenic compounds has been few reported in
369 France as compared to other industrialized countries. Nonetheless, effluents from sewage
370 water treatment plant (SWTP) have been shown to be the source of steroid estrogens at
371 different locations (Labadie and Budzinski, 2005, Muller et al., 2008; Cargouet et al., 2004);
372 in surface waters, natural and synthetic estrogens were also described as main estrogenic
373 chemicals in the Seine river downstream Paris, France (Cargouet et al., 2004). In sediments
374 sampled downstream from a large SWTP in the Seine river, Fenet et al. (2003) reported that
375 alkylphenols were the main contributors to the estrogenic activities detected by MELN
376 bioassay while steroid estrogens were not measured in their study. This contrasts with the
377 very low contribution of alkylphenols measured in the present study where estrogenic
378 activities were predominantly explained by the natural estrogens βE2 and E1. Since
379 alkylphenols are often considered as tracer compounds of water treatment effluents, the low
380 levels measured in our study could reflect the fact that our sites were not directly impacted by
381 SWTP effluents, the main source of aquatic pollution by alkylphenols. Nevertheless, other
382 studies in European countries have reported occurrence of steroid estrogens in river sediments
383 at similar levels than those found in our study, i.e. in the 0.3-5 ng/g range (Houtman et al.,

384 2006; Labadie and Hill, 2007; Reddy et al., 2005; Matějíček et al., 2007). Moreover, the large
385 contribution of natural estrogens to biological E2-EQs is in agreement with the study of
386 Houtman et al. (2006) who reported that E1 and βE2 were responsible for more than 75 % of
387 the estrogenic activity in sediments from Zierikze harbour, The Netherlands. In the low
388 impacted sites (Aisne, Vallon du Vivier and Lézarde), natural estrogens nearly explained 100
389 % of the estrogenic activity detected by the bioassay. Conversely, in Réveillon and Rhonelle
390 samples, only a part (28 and 35 %) of the estrogenic activity was explained by βE2 and E1,
391 suggesting the presence of other xeno-estrogens in the samples. The Réveillon River is under
392 pressure of multiple sources of contamination and was indeed found to be contaminated by
393 many organic chemicals from agricultural (pesticides) and urban/industrial (BPA,
394 alkylphenols, parabens) origins. However, the targeted xeno-estrogens could not explain the
395 overall estrogenic of the sample, hence suggesting the presence of other ER ligands that were
396 not included in our analyses. This stresses the limits of the Chem-EQ based approach to
397 identify bioactive compounds in complex mixtures and argues for further investigations, for
398 instance by using effect directed analysis (EDA) approaches based on sample fractionation
399 and identification of bioassay active fractions (Brack, 2003), in order to better characterize the
400 contamination of this site by EDCs.

401 ***4.3. (Anti)androgenic activities***

402 The occurrence of (anti)androgenic compounds in river sediments has been rarely reported as
403 compared to estrogenic or dioxin-like compounds. However, such compounds can be present
404 in river sediment since high levels of androgenic activities (1-15 ng DHT-EQ/g) were
405 quantified by using the YAS assay in sediment from United Kingdom estuaries (Thomas et
406 al., 2002). Natural androgenic steroids in sewage treatment effluents were identified as
407 possible source of contamination (Thomas et al., 2002). Recently, Urbatzka et al. (2007)
408 reported both androgenic and antiandrogenic activities in fractionated sediment extracts from

409 the river Lambro, Italy, but the chemicals responsible for androgenicity in YAS assay were
410 not identified. Other studies have shown that pulp mill effluents (Jenkins et al., 2004) or
411 livestock feedlot effluents (Soto et al., 2004) are potential sources for androgens in the aquatic
412 environment. In our study, we detected androgenic activity (4 ng DHT-EQ/g) in Rhonelle
413 river sediment but the responsible compounds remain to be identified. In addition,
414 antiandrogenic activities were detected in three other sites (Rév, Lez and VdV). Although
415 several antiandrogenic chemicals were detected at these sites (i.e. BHT, BPA, alkylphenols,
416 pesticides, Table 5), the measured concentrations could explain only a minor part of the
417 activities measured in the MDA-kb2 bioassay. Therefore, the later were likely due to the
418 presence of other EDCs that have not been investigated in our chemical analyses.

419 ***4.4. PXR activities***

420 To our knowledge, the present study is the first demonstration of PXR-mediated activity in
421 river sediments. The human PXR is known to be activated by a large panel of environmental
422 chemicals that belong to different classes, like pharmaceuticals, steroids, alkylphenols (Mnif
423 et al., 2007), polybrominated diphenyl ethers (Pacyniak et al., 2007), as well as various
424 pesticides (Lemaire et al., 2006). In the present study, human PXR activating substances were
425 detected in all analyzed samples, at concentrations in the µg/g range in terms of SR12813-
426 EQs. Since PXR shares several ligands with the estrogen receptor (ER), it was hypothesized
427 that several of the (xeno)estrogens detected in our samples could have contributed to the
428 PXR-mediated activity. However, the Chem-SR12813-EQ/Bio-SR12813-EQ ratios showed
429 that, at the measured concentrations, analyzed (xeno)estrogens only poorly contributed to the
430 SR12813-EQ quantities determined by the bioassay. Thus, more investigations using
431 fractionation and isolation procedures will be necessary to characterize the compounds
432 responsible for PXR activation in sediments. One promising methodology will likely consist
433 in the use of purified PXR immobilized on columns in order to isolate PXR ligands from

complex mixtures (Pillon et al., 2005; Balaguer et al., unpublished). It is expected that such approach will allow identifying environmental PXR ligands and thus providing further useful information on the toxicological relevance of detection of PXR activity in aquatic ecosystems.

4.5. Comparison with fish biomarkers

In the present study, toxicological profiling of sediments showed the presence of a wide range of chemicals that could potentially affect different molecular targets, namely ER, AR, AhR and PXR, involved in the regulation of the endocrine system of exposed organisms. At the studied sites, recent biomarker studies in wild three-spine stickleback (*Gasterosteus aculeatus*) revealed fish exposure to different chemical stress including endocrine disrupters and dioxin-like compounds (Sanchez et al., 2007, Sanchez et al., 2008). Although statistical correlation between fish biomarker and *in vitro* bioassays could not be tested because of the small number of sites, some concordances between the two approaches were noted. For instance, vitellogenin induction in male stickleback has been evidenced in Réveillon and Rhonelle rivers, which were the most active in the MELN assay (Table 4). In addition, female stickleback from the Rhonelle River abnormally produced elevated levels of spigging, a male glue protein synthesized in the kidney and used for building nest (Sanchez et al., 2008). This suggested exposure to androgenic compounds, which correlates with the finding of AR-mediated activity in MDA-kb2 cells in our study (Figure 4a). For dioxin-like activities, significant EROD induction in male and female stickleback was reported in Réveillon and Lézarde (Sanchez et al., 2007), the most active samples in the PLHC-1 assay (Table 3). On the whole, the bioanalytical approach confirmed the multipollution context at impacted sites like Réveillon and Rhonelle, and provided new information on possible causal agents for abnormal endocrine responses in fish. Although we identified some of the chemicals responsible for *in vitro* activities in sediment extracts, the direct extrapolation to fish exposure is rather risky since it may depend on site specific characteristics that can influence pollutant

partitioning between sediment, suspended matter and dissolved phase in the water column. Thus, more investigation using appropriate sampling methods like passive samplers will be needed in order to link chemical contamination by EDCs and fish exposure and effects.

5. Conclusion

In summary, this study reports for the first time the simultaneous assessment of multiple endocrine active and dioxin-like chemicals, in French river sediments sampled in small streams subjected to various diffuse pollutions. Besides the major contribution of natural steroids and PAH-like compounds to estrogenic and dioxin-like activities, (anti)androgenic and PXR-mediated activities were detected although the individual active compounds could not be identified using targeted chemical analyses. These samples are thus interesting candidates for further EDA studies, which are under progress in order to elucidate the causal agents.

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Figure legends

Figure 1. Doses-response curves for EROD induction by sediment organic extracts in PLHC-1 cells measured after (a) 4h and (b) 24h exposure periods. Numbers correspond to the different sites, with 1: Lez, 2: Rev, 3: Rho, 4: VdV, 5: Ais. Values represent the mean ± SD, n=3.

Figure 2. Linear regression (log scale) showing correlation between PLHC-1 bioassay- (Bio-) and PAH chemical analyses- (Chem-) derived BaP-EQs (a) and TCDD-EQs (b) in sediment samples.

Figure 3. Estrogenic activity of (a) 17β-estradiol and (b) the five sediment extracts (1: Rev, 2: Rho, 3: VdV, 4: Ais, 5: Lez) in MELN cells. Results are expressed as percentage of maximal luciferase activity induced by β-E2 at 10 nM. Values are means of triplicates ± SD.

Figure 4. (a) Androgenic and (b) anti-androgenic activities of the reference chemicals [dihydrotestosterone (DHT) and Flutamide (Flu)] and sediment extracts in MDA-kb2 cells. Results are expressed as percentage of the maximal luciferase activity induced by DHT at (a) 10 nM and (b) 0.3 nM. Values are means of triplicates ± SD.

Figure 5. (a) PXR-mediated dose-response curves of reference chemical (SR 12813) and sediment extracts (1: Rev, 2: Rho, 3: Lez, 4: VdV, 5: Ais) in HG_5LN-PXR cells. Results are expressed as percentage of maximal luciferase activity induced by SR 12813 at 0.3 µM. (b) Non specific effect of sediment extracts on constitutive luciferase expression in HG_5LN cells. Results are expressed as percentage of luciferase activity in control cells. Values are means of triplicates ± SD.

659 Table 1. Summary of some general characteristics of sampling sites
660

Sites	Aisne (Ais)	Vallon du Vivier (VdV)	Rhonelle (Rho)	Réveillon (Rev)	Lézarde Lez)
GPS coordinates	N 49°23'55" E 3°28'31"	N 49°43'23" E 0°27'42"	N 50°17'49" E 3°32'41"	N 48°34'00" E 2°32'09"	N 49°34'09" E 0°13'20"
Pressures[a]	Low	Mixed	Urban dense	Urban dense	Mixed
Water quality[b]	Good	Very good	Bad	Very bad	Good
Biomarker responses in fish (*Gasterosteus aculeatus*)[c]	No data	No alteration (reference site)	Induction of vitellogenin in male and spigging in female	Induction of EROD and vitellogenin in male	Induction of EROD

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662
663 a, Data obtained from the Rivers Waterbase of the European Environmental Agency (EEA,
664 2008); b, data from obtained from French water agencies; c, data from Sanchez et al. (2008).
665

666 Table 2. Overview of investigated chemicals: chemical families, analytical standard sources
667 and methods used for their quantification in samples
668

Classes (Providers)	Chemicals	Analytical methods (Reference)
Natural and synthetic estrogens (Sigma-Aldrich)	estrone (**E1**), 17β-estradiol (**17β-E2**), 17α-estradiol (**17α-E2**), 17α-ethynilestradiol (**EE2**), estriol (**E3**), mestranol (**MeEE2**), diethylstilbestrol **(DES**)	GC/MS with derivatization (Kinani et al., (2008a))
Alkylphenols (Sigma-Aldrich)	4-n-nonylphenol (**4-n-NP**), 4-tert-octylphenol (**4-t-OP**), 4-n-butoxyphenol (**4-BuOP**), 4,4'-isopropylidene diphenol (**bisphenol A, BPA**)	GC/MS/MS with derivatization (Kinani et al., (2008a))
Parabens (Sigma-Aldrich)	n-propylparaben (**PrP**), n-butylparaben (**BP**), benzylparaben (**BzP**)	GC/MS and GC/MS/MS with derivatization (Kinani et al., (2008a))
hydroxy-PAHs (Sigma-Aldrich)	1-hydroxypyrene (**1-OHPyr**), 2-hydroxyfluorene (**2-OHFlu**)	GC/MS/MS with derivatization (Kinani et al., (2008a))
PAHs (Sigma-Aldrich)	naphthalene (**Nap**), acenaphthylene (**Acy**), acenaphthene (**Ace)**, fluorene (**Flu**), phenanthrene (**Phe**), anthracene (**Ant**), fluoranthene (**Flt**), pyrene (**Pyr**), benz[*a*]anthracene (**B[a]A**), chrysene (**Chr**), benzo[b]fluoranthene (**B[b]F**), benzo[k]fluoranthene (**B[k]F**), benzo[*a*]pyrene (**B[a]P**), indeno[1,2,3-c,d]pyrene (**Ind**), dibenz[a,h]anthracene (**DBA**), benzo[g,h,i]perylene (**B[ghi]P**)	GC/MS (Louiz et al., 2008)
Organochlorine pesticides (Promochem)	hexachlorobenzene (**HCB**), lindane (**γ-HCH**), vinclozolin, metolachlor, endosulfan (2α:1β), *o,p'*-DDT, methoxychlor, fenarimol	GC/MS and GC/MS/MS see section 2.5.1
Phytoestrogens (Sigma-Aldrich)	daidzein, genistein, biochanin A, equol, coumestrol, resveratrol	LC/MS/MS see section 2.5.2
Mycoestrogens (Sigma-Aldrich)	zearalenone (**ZON**), α-zearalenol (**α-ZOL**), β-zearalenol (**β-ZOL**), zearalanone (**ZEA**), α-zearalanol (**α-ZAL**) and β-zearalanol (**β-ZAL**)	GC/MS with derivatization (Kinani et al., (2008b))

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Table 3. Concentrations of the 16 PAHs and their relative contribution to the total dioxin-like activity in sediment organic extracts from the five studied sites.

Chemicals	LOQ [a] (ng/g)	IEF [b]		Concentration in sampling sites in ng/g d.w.				
		BaP 4h	TCDD 24h	Ais	VdV	Rho	Rev	Lez
Nap	0.11	n.i.[c]	n.i.	n.d.[d]	3.21	2.30	5.42	3.73
Acpy	0.17	5.56E-3	n.i.	n.d.	3.57	15.28	54.96	77.27
Acp	0.08	n.i.	n.i.	n.d.	4.94	2.56	26.18	38.66
Flu	0.09	1.44E-2	n.i.	n.d.	9.01	8.74	38.97	73.46
Phe	0.06	n.i.	n.i.	10.67	132.66	96.61	612.83	1706.67
Ant	0.11	n.i.	n.i.	3.30	37.44	46.01	225.27	712.69
Flt	0.05	n.i.	n.i.	46.35	307.42	267.01	1533.00	3367.66
Pyr	0.17	3.58E-3	3.85E-5	36.11	231.11	168.45	1307.67	2907.47
B[a]A	0.06	2.58E-1	9.77E-5	11.14	110.79	150.53	761.47	1477.33
Chr	0.06	2.92E-1	3.76E-4	8.02	102.24	172.16	1013.47	1596.00
B[b]F	0.13	6.94E-1	4.63E-4	15.59	69.66	723.05	2760.67	4674.00
B[k]F	0.11	2.94	4.23E-3	4.14	57.38	370.71	1602.09	2681.37
B[a]P	0.20	1	5.13E-4	15.26	97.99	138.03	841.60	1685.49
Ind	0.07	8.43E-1	1.64E-3	35.76	171.62	116.08	723.40	1190.89
DBA	0.12	3.66	6.11E-3	1.97	8.38	16.62	45.09	98.72
B[ghi]P	0.16	n.i.	n.i.	21.00	115.42	71.09	423.98	786.24
Sum-PAHs (ng/g d.w.)				*209.3*	*1462.8*	*236.5*	*11976.1*	*23077.7*
Chem-BaP-EQ (ng/g d.w.)				81	550	1978	8740	15037
Bio-BaP-EQ (ng/g d.w.)				200	910	7334	31556	75435
Chem-BaP-EQ / Bio-BaP-EQ (%)				*41*	*60*	*27*	*28*	*20*
Chem-TCDD-EQ (ng/g d.w.)				0.11	0.72	2.35	10.45	17.78
Bio-TCDD-EQ (ng/g d.w.)				0.67	4.49	5.89	38.43	48.38
Chem-TCDD-EQ / Bio-TCDD-EQ (%)				*16*	*16*	*40*	*27*	*37*

[a] LOQ: limits of quantification provided by the GC-MS method, [b] IEF: induction equivalent factors relative to BaP after 4 h and to TCDD after 24 h of exposure (from Louiz et al., 2008), [c] n.i.: no EROD induction detected within the 0.1 nM to 10 µM concentration range, [d] n.d: not detected or below quantification limit.

680 Table 4. Concentrations of estrogenic compounds measured in sediment organic extracts from
681 the five studied sites, and their relative contribution to the total estrogenic activity measured
682 in the MELN bioassay.
683

Chemical classes	Chemicals	EEF[a]	LOQ[b] (ng/g)	Concentration in sediment extracts (ng/g d.w.)				
				Ais	VdV	Rho	Rev	Lez
Natural estrogens	α-E2	0.02	0.04	0.04	n.d.	0.2	n.d.	n.d.
	β-E2	1	0.05	0.27	0.78	0.58	1.58	0.18
	E1	0.02	0.02	0.3	0.58	0.48	1.28	0.36
	E3	0.17	0.6	n.d.	n.d.	n.d.	1.26	n.d.
Synthetic estrogens	EE2	0.93	0.07	n.d.	n.d.	n.d.	n.d.	n.d.
	MeEE2	0.02	0.08	n.d.	n.d.	n.d.	n.d.	n.d.
	DES	0.17	0.03	n.d.	n.d.	n.d.	n.d.	n.d.
Alkylphenols	4-t-OP	1.1E-04	0.03	n.d.	n.d.	0.56	6.3	n.d.
	4-n-NP	3.3E-06	0.01	n.d.	0.52	n.d.	0.04	n.d.
	BPA	4.5E-05	0.01	2.29	11.67	7.92	47.28	1.24
Parabens	PrP	7.4E-06	<0.01	n.d.	n.d.	n.d.	0.11	n.d.
	BuP	4.9E-06	<0.01	n.d.	n.d.	n.d.	0.07	n.d.
	BzP	6.6E-06	0.02	n.d.	n.d.	n.d.	0.79	n.d.
PAHs metabolites	1OHPyr	9.9E-07	0.02	0.11	0.26	0.3	0.41	0.37
	2OHFlu	6.3E-06	0.02	n.d.	n.d.	0.19	0.3	0.6
Pesticides	Endosulfan(α)	2.0E-06	0.21	n.d.	n.d.	n.d.	63.84	n.d.
	Endosulfan(β)	2.0E-06	1.25	n.d.	n.d.	n.d.	29.2	n.d.
	o,p'-DDT	1.7E-05	0.22	6.52	n.d.	n.d.	7.25	n.d.
Phytoestrogen	Equol	2.8E-04	0.15	n.d.	n.d.	0.17	n.d.	n.d.
Mycoestrogens	α-ZAL	0.14	0.13	n.d.	n.d.	n.d.	< LOQ	n.d.
	β-ZAL	0.03	0.02	n.d.	n.d.	n.d.	< LOQ	n.d.
Chem-E2-EQ (ng/g) [d]			-	0.28	0.79	0.59	1.82	0.19
Bio-E2-EQ (ng/g) [e]			0.010	0.29	0.83	1.69	6.43	0.20
Chem-E2-EQ / Biol-E2-EQ (%)				96	95	35	28	94

684
685 [a] EEF: estradiol equivalence factors relative to estradiol, determined as described in the Materials and Methods
686 section, [b] LOQ: limit of quantification, [c] n.d.: not detected or below quantification limits, [d] Chem-E2-EQs:
687 chemical estradiol equivalents, [e] Biol-E2-EQs: biological estradiol equivalents based on EC_{25} effective
688 concentration in the MELN bioassay.
689

690 Table 5. Summary of anti-androgenic and PXR-mediated activities in sediment extracts and
691 relative contribution of known anti-androgenic and PXR ligands measured in organic extracts
692 from the five studied sites.
693

Chemicals	FEF[a]	SREF[a]	LOQ[b] (ng/g)	Concentration at sampling sites (ng/g sed d.w)				
				Ais	VdV	Rho	Rev	Lez
BPA	0.394	0.025	0.01	2.29	11.67	7.92	47.28	1.24
4-t-OP	0.339	0.099	0.03	n.d.	n.d.	0.56	6.3	n.d.
BHT	0.101	n.a.	0.07	n.d.	3.13	3.6	14.14	0.61
4-n-NP	0.016	n.a.	0.01	n.d.	0.52	n.d.	0.04	n.d.
Endosulfan(α)	0.058	0.029	0.21	n.d.	n.d.	n.d.	63.84	n.d.
o,p'-DDT	0.15	0.032	0.22	6.52	n.d.	n.d.	7.25	n.d.
Vinclozolin	2.68	n.a.	0.24	4.57	n.d.	n.d.	n.d.	n.d.
β-E2	n.a.	0.129	0.05	0.27	0.78	0.58	1.58	0.18
E1	n.a.	0.13	0.02	0.3	0.58	0.48	1.28	0.36
Chem-Flu-EQ (ng/g)				14.1	4.9	3.7	27.0	0.6
Bio-Flu-EQ (ng/g)[d]			17.0	nd	1089.4	nd	32493.4	7444.9
Chem-Flu-EQ / Biol-Flu-EQ (%)				-	0.45	-	0.08	0.01
Chem-SR12813-EQ (ng/g)[d]				0.34	0.47	0.39	4.26	0.10
Bio-SR12813-EQ (ng/g) [d]			40.0	964.8	1647.2	14322.2	51317.3	2147.1
Chem- SR12813-EQ / Biol- SR12813-EQ (%)				0.035	0.028	0.003	0.008	0.005

694
695 [a] FEF (Flutamide equivalent factors) were determined as described in Materials and Methods; SREF
696 (SR12813 equivalent factors) were from Lemaire et al. (2004) for endosulfan and from Mnif et al.
697 (2007) for all other compounds. [b] LOQ: limit of quantification. n.a.: non active compound, n.d. not
698 detected.
699