

1 **Bioanalytical characterisation of multiple endocrine- and dioxin-like activities in**  
2 **sediments from reference and impacted small rivers**

3

4 Said Kinani<sup>a,b,\*</sup>, Stéphane Bouchonnet<sup>b,\*</sup>, Nicolas Creusot<sup>a</sup>, Sophie Bourcier<sup>b</sup>, Patrick  
5 Balaguer<sup>c</sup>, Jean-Marc Porcher<sup>a</sup> and Sélim Aït-Aïssa<sup>a,1,\*</sup>

6

7 <sup>a</sup> Unité d'Ecotoxicologie, Institut National de l'Environnement Industriel et des Risques  
8 (INERIS), BP2, F-60550 Verneuil en Halatte, France.

9 <sup>b</sup> Département de Chimie des Mécanismes Réactionnels, Ecole Polytechnique, 91128  
10 Palaiseau Cedex, France.

11 <sup>c</sup> Institut National de la Santé et de la Recherche Médicale (INSERM), U896, Montpellier, F-  
12 34298, France.

13

14 1 : Corresponding author: Tel +33 344 556 511, Fax +33 344 556 767

15 \* E-mail addresses : selim.ait-aïssa@ineris.fr (Sélim Aït-Aïssa), said@dcmr.polytechnique.fr  
16 (Said Kinani), stephane.bouchonnet@dcmr.polytechnique.fr (Stéphane Bouchonnet).

17

## 18 **Abstract**

19 A comprehensive evaluation of organic contamination was performed in sediments sampled  
20 in two reference and three impacted small streams where endocrine disruptive (ED) effects in  
21 fish have been evidenced. The approach combined quantitative chemical analyses of more  
22 than 50 ED chemicals and *in vitro* bioassays allowing the quantification of receptor-mediated  
23 activities, namely estrogen (ER), androgen (AR), dioxin (AhR) and pregnane X (PXR)  
24 receptors. At the most impacted sites, chemical analyses showed the presence of natural  
25 estrogens, organochlorine pesticides, parabens, polycyclic aromatic hydrocarbons (16 PAHs),  
26 bisphenol A and alkylphenols, while synthetic steroids, myco-estrogens and phyto-estrogens  
27 were not detected. Determination of toxic-equivalent amounts showed that 28 to 96% of  
28 estrogenic activities in bioassays (0.2-6.3 ng/g 17 $\beta$ -estradiol equivalents) were explained by  
29 17 $\beta$ -estradiol and estrone. PAHs were major contributors (20-60%) to the total dioxin-like  
30 activities. Interestingly, high PXR and (anti)AR activities were detected; however, analysed  
31 compounds could not explain the measured biological activities.

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

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

36

## 37 **1. Introduction**

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

48 Up to date, most attention has been directed to identifying estrogenic chemicals, i.e. those  
49 capable of binding to and activating the estrogen receptor (ER), because many of the effects  
50 reported in wildlife appear to be a consequence of feminization of males (Sumpter et al.,  
51 2005). However, many environmental contaminants can interfere with other molecular targets  
52 involved in the regulation of the endocrine system. These include other nuclear receptors like  
53 androgen receptor (AR), pregnane X receptor (PXR, e.g. Mnif et al., 2007) as well as  
54 steroidogenesis enzymes like aromatase (e.g. Laville et al., 2006). EDCs can also act via  
55 indirect mechanisms such as (anti)estrogenic effect mediated by aryl hydrocarbon receptor  
56 (AhR) ligands through an ER/AhR cross-talk dependent mechanism (Othake et al., 2003,  
57 2007). Therefore, a comprehensive assessment of EDC hazard should take into account the  
58 ability of chemicals to interfere with different targets of the endocrine system (Houtman et al.,  
59 2004).

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

62 complex environmental mixtures (Eggen and Segner, 2003). In particular, *in vitro* assays  
63 using reporter gene activation in stably transfected cell lines provide robust, sensitive and  
64 specific bioassays to screen and quantify endocrine activities in environmental samples.  
65 Combining such tools with powerful chemical analyses within integrated approaches permits  
66 to identify key toxicants to be monitored in the environment (Brack, 2003). By using such  
67 bioanalytical approaches, the occurrence of EDCs and their effects to aquatic organisms has  
68 been several times reported in different industrialised countries like the United Kingdom (e.g.  
69 Jobling et al., 2006) or the Netherlands (e.g. Vethaak et al., 2005). However in France, much  
70 fewer data have been reported. Recent studies showed occurrence of steroid estrogens and  
71 alkylphenols in effluents from sewage treatment plants (Labadie and Budzinski, 2005, Muller  
72 et al., 2008) or in water and sediment from the Seine River (Fenet et al., 2003; Cargouet et al.,  
73 2004). More recently, abnormal elevated levels of vitellogenin and spigging, used as  
74 biomarkers of estrogen and androgen exposure respectively, have been reported in three-spine  
75 stickleback (*Gasterosteus aculeatus*) sampled in different small streams subjected to diffuse  
76 pollution like agricultural run-off or domestic effluents (Sanchez et al., 2008). Thus, more  
77 investigation is needed in order to characterise and identify key EDCs in French aquatic  
78 ecosystems.

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

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

90

## 91 **2. Materials and methods**

92

### 93 **2.1. Chemicals and reagents**

94 List, abbreviation and source of analytical standards are given in Table 2. Flutamide (Flut), 3-  
95 (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide tetrazolium (MTT), 5 $\alpha$ -  
96 dihydrotestosterone (DHT), as well as the analytical internal standards including [<sup>13</sup>C<sub>6</sub>]4-n-  
97 nonylphenol, [<sup>2</sup>H<sub>2</sub>]17 $\beta$ -estradiol, [<sup>2</sup>H<sub>10</sub>]phenanthrene, [<sup>2</sup>H<sub>10</sub>]acenaphthene, [<sup>2</sup>H<sub>12</sub>]perylene and  
98 [<sup>2</sup>H<sub>12</sub>]chrysene were purchased from Sigma-Aldrich (St Quentin Fallavier, France). ICI  
99 182,780 (ICI) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were obtained from Tocris  
100 Bioscience (Ellisville, USA) and Promochem (Molsheim, France), respectively. All standards  
101 were of 98.1–99.9% purity. Chromatographic-grade solvents: acetone, methanol, acetonitrile,  
102 dimethylsulfoxide (DMSO) and n-hexane were also purchased from Sigma-Aldrich. Stock  
103 solutions of  $\beta$ -E2 2.72 g/L, DHT 2.90 g/L and B[a]P 2.52 g/L were prepared by dissolving  
104 crystalline compound in DMSO. A solution of TCDD was prepared by drying an aliquot of  
105 TCDD in methanol under a nitrogen stream and dissolving the residue in DMSO to reach a  
106 concentration of 3.22 g/L.

107

### 108 **2.2. Site description and sampling**

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

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

119

### 120 **2.3. Extraction procedure**

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

133

### 134 **2.4. Cell cultures and in vitro bioassays**

135 **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<sub>5</sub>LN-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- $\beta$ Glob-  
139 Luc-SVNeo plasmid (Balaguer et al., 2001). The HG<sub>5</sub>LN-PXR cell line was derived from  
140 HeLa cells that were first stably transfected with GAL4RE<sub>5</sub>-bGlob-Luc-SV-Neo (HG<sub>5</sub>LN  
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<sub>2</sub> 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  $\mu$ L 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 ( $\mu$ Beta, 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  $\mu$ M,  
160 DHT 10 nM (for AR agonist activity) and DHT 0.3 nM (for AR antagonist activity) in

161 MELN, HG<sub>5</sub>LN-PXR and MDA-kb2 cell lines, respectively. For ER and AR agonistic  
162 responses, the specificity of luciferase induction by samples was checked by co-exposure  
163 experiments with 0.1 µM ICI182,780 or 1 µM flutamide, as reference ER and AR antagonists,  
164 respectively.

#### 165 *2.4.2. Dioxin-like activity in PLHC-1 cell line*

166 The fish hepatic PLHC-1 cell line (ATCC, #CRL-2406) was described by Ryan and  
167 Hightower (1994). The cells were routinely grown at 30 °C in E-MEM culture media  
168 supplemented with 10 % FCS and 1 % antibiotics in a 5 % CO<sub>2</sub> humidified atmosphere. For  
169 experiments, cells were seeded in 96-well plates at a density of approximately 50 000 cells  
170 per well. After 24 hours of incubation, cells were exposed to test chemicals or sample extracts  
171 for either a 4 h or 24 h incubation period, in order to differentiate between dioxin-like  
172 compounds that are rapidly metabolized (e.g. PAHs) in the cells and dioxin-like chemicals  
173 that are persistent in the cells (e.g. dioxins) (Louiz et al., 2008). Then, plates were processed  
174 for 7-ethoxyresorufin-O-deethylase (EROD) activity in intact cells, following the protocol  
175 previously described by Laville et al. (2004). Results were expressed as percent of EROD  
176 activity induced by the positive control (TCDD 1 nM).

#### 177 *2.4.3. Cell viability*

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

181

### 182 *2.5. Chemical analyses*

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

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

#### 190 2.5.1. GC-MS and GC-MS/MS analyses

191 The chromatographic separation was performed with a 60 m “Factor four VF-10-MS” (10%  
192 phenyl, 90 % methylpolysiloxane) capillary column (internal diameter: 0.25 mm, film  
193 thickness: 0.25  $\mu$ m) from Varian. All experiments were performed by automatically injecting  
194 2.0  $\mu$ L of sample in the splitless mode at a rate of 50  $\mu$ L/s. Helium (99.999% purity) was used  
195 as the carrier gas at a constant flow of 1.0 mL/min for PAHs analysis and at 1.4 mL/min for  
196 the other target compounds; and was held a constant flow by electronic pressure control. The  
197 injector temperature was set to 300 °C. The split valve opened after 1 min, with a split ratio of  
198 35/100. The manifold, ion trap source and transfer line temperatures were set to 120, 220 and  
199 300 °C, respectively.

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

#### 210 2.5.2. LC-MS/MS analyses

211 LC/MS is equipped with an electrospray ionization (ESI) source operated in negative-ion  
212 mode. The analytical column used was a Pursuit C18 (150 mm x 2.1 mm I.D., 5µm particle  
213 size, Varian, France) and a mobile phase consisting of acetonitrile (A) and water (B). The  
214 gradient conditions were as followed: 25% of (A) and 75% of (B) for 2 min, followed by a  
215 linear increase to 75% (A) at 16 min and 2 min hold at 75% (A). The flow rate was 0.2  
216 mL/min and the injection volume was 20 µL. Only phytoestrogens were analysed by LC-MS.  
217 The mass spectrometer was operated in order to record the MS/MS spectrum of deprotonated  
218 pseudomolecular ion  $[M-H]^-$ . Collisions energies have been optimized between 20 to 30 eV  
219 for each compound to obtain three characteristic ions. The optimized instrument conditions  
220 were as followed: capillary voltage -3.4 kV, cone voltage, 50 V; multiplier voltage, 2250 V;  
221 source temperature, 100°C; desolvation temperature, 450°C. Nitrogen was used as both the  
222 nebulizing (50 L/h) and desolvation gas (700 L/h). Argon was used as collision gas at flow  
223 0.28 (mL/min).

224

## 225 ***2.6. Data analysis and determination of bioassay- and instrumental-derived toxic-***

### 226 ***equivalents***

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

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

238 In order to estimate the contribution of analysed compounds to the total activity detected by a  
239 bioassay, the measured concentrations (as ng/g sed wt) were converted to toxic-equivalent  
240 activities derived from chemical analysis (Chem-ref-EQs), that is Chem-BaP-EQ, Chem-  
241 TCDD-EQ, Chem-E2-EQ, Chem-DHT-EQ, Chem-Flu-EQ and Chem-SR12813-EQ, in each  
242 respective bioassay. The Chem-ref-EQ were calculated according to the following equation:  
243  $\text{Chem-ref-EQ} = \sum (C_i \times \text{ref-EF}_i)$ , where, for a given chemical  $i$ ,  $C_i$  is the measured  
244 concentration in a sample and  $\text{ref-EF}_i$  is the inducing/inhibiting equivalent factor relative to  
245 the reference ligand (i.e. BaP, TCDD, E2, DHT, Flu or SR12813) in a given bioassay. The  
246 equivalent factors were calculated as follows:  $\text{ref-EF}_i = \text{EC}_{25} \text{ of reference compound} / \text{EC}_{25} \text{ of}$   
247  $\text{test compound } i$ , on mass basis. Unless otherwise specified in Tables 3-5, ref-EFs of each  
248 analysed compound were determined experimentally in each bioassay by establishing dose-  
249 response curves for individual standard chemicals and by comparing them to that of the  
250 reference compound. For instance, the 21 individual molecules listed in Table 4 were tested  
251 for their ability to induce luciferase activity in MELN cells in our assay condition, and their  
252 E2-equivalent factors (EEF) were then determined and used to calculate Chem-E2-EQ in the  
253 samples. Finally, the ratio Chem-ref-EQ/Bio-ref-EQ allowed evaluating the contribution of  
254 quantified compounds to the biological activity detected by the bioassay, i.e. a ratio near  
255 100% means that all analysed compounds were explicative for biological results; otherwise  
256 other non analysed compounds may be present in the samples.

257

## 258 **3. Results**

### 259 ***3.1. Dioxin-like activity in river sediment extracts***

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

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

276 The results given in Figure 2 showed that chemical- and bioassay-derived BaP-EQs and  
277 TCCD-EQs were highly correlated ( $r^2 = 0.99$  and  $0.97$ , respectively), which confirmed the  
278 involvement of PAHs in the detected biological activities at both exposure durations.

279 However, in all sediment extracts, the Chem-EQs were significantly lower than the Bio-EQs,  
280 indicating that only a part of activity was explained by analyzed PAHs, which accounted for  
281 approximately 20 to 60 % and 16 to 40 % of the BaP-EQs and TCCD-EQs measured in  
282 PLHC-1, respectively.

283

284 **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  $\beta$ -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  $\beta$ -E2, the use of EC<sub>25</sub> 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,  $\beta$ -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  $\alpha$ -ZAL and  $\beta$ -ZAL.  $\alpha$ -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  $\beta$ -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<sub>5</sub>LN cell line, which  
327 expresses only the GAL4-luciferase construct (Figure 5b). In HG<sub>5</sub>LN 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.

334  
335  
336  
337  
338  
339  
340  
341  
342  
343  
344  
345  
346  
347  
348  
349  
350  
351  
352  
353  
354  
355  
356  
357  
358

## 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  $\beta$ E2 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  $\mu\text{g/g}$  of dry sediment at various sites from the Seine estuary (Cachot et al., 2006), and from 2.3 to 41.3  $\mu\text{g/g}$  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  $\beta$ E2 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; Matějčiček 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  $\beta$ E2 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  $\beta$ E2 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 (R ev, 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  $\mu\text{g/g}$  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

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

#### 437 ***4.5. Comparison with fish biomarkers***

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

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

## 462 **5. Conclusion**

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

## 472 **Acknowledgements**

473 The authors wish to thank Emmanuelle Maillot-Maréchal for excellent technical help with the  
474 cell cultures. This study was funded by the French Ministry of Ecology and Sustainable  
475 Development (Program 189), the “Agence Française de Sécurité Sanitaire de l’Environnement  
476 et du Travail” (AFSSET, RD-2005-02) and by a doctoral fellowship from the ANRT and  
477 INERIS (to SK).

## 479 **5. References**

480 Balaguer, P., Boussioux, A.M., Demirpence, E., Nicolas, J.C., 2001. Reporter cell lines are  
481 useful tools for monitoring biological activity of nuclear receptor ligands. *Luminescence*  
482 16, 153-158.

- 483 Brack, W., 2003. Effect-directed analysis: a promising tool for the identification of organic  
484 toxicants in complex mixtures? *Analytical and Bioanalytical Chemistry* 377, 397-407.
- 485 Brack, W., Schirmer, K., Erdinger, L., Hollert, H., 2005. Effect-directed analysis of mutagens  
486 and ethoxyresorufin-O-deethylase inducers in aquatic sediments. *Environmental*  
487 *Toxicology and Chemistry* 24, 2445-2458.
- 488 Cachot, J., Geffard, O., Augagneur, S., Lacroix, S., Le Menach, K., Peluhet, L., Couteau, J.,  
489 Denier, X., Devier, M.H., Pottier, D., Budzinski, H., 2006. Evidence of genotoxicity  
490 related to high PAH content of sediments in the upper part of the Seine estuary  
491 (Normandy, France). *Aquatic Toxicology* 79, 257-267.
- 492 Cargouet, M., Perdiz, D., Mouatassim-Souali, A., Tamisier-Karolak, S., Levi, Y., 2004.  
493 Assessment of river contamination by estrogenic compounds in Paris area (France).  
494 *Science of the Total Environment* 324, 55-66.
- 495 Eggen, R.I.L., Segner, H., 2003. The potential of mechanism-based bioanalytical tools in  
496 ecotoxicological exposure and effect assessment. *Analytical and Bioanalytical Chemistry*,  
497 377, 386-396.
- 498 Fenet, H., Gomez, E., Pillon, A., Rosain, D., Nicolas, J.C., Casellas, C., Balaguer, P., 2003.  
499 Estrogenic activity in water and sediments of a French river: contribution of alkylphenols.  
500 *Archives of Environmental Contamination and Toxicology* 44, 1-6.
- 501 Hartmann, N., Erbs, M., Wettstein, F.E., Schwarzenbach, R.P., Bucheli, T.D., 2007.  
502 Quantification of estrogenic mycotoxins at the ng/L level in aqueous environmental  
503 samples using deuterated internal standards. *Journal of Chromatography A*, 1138, 132-  
504 140.
- 505 Hollert, H., Durr, M., Olsman, H., Halldin, K., Van Bavel, B., Brack, W., Tysklind, M.,  
506 Engwall, M., Braunbeck, T., 2002. Biological and chemical determination of dioxin-like

507 compounds in sediments by means of a sediment triad approach in the catchment area of  
508 the River Neckar. *Ecotoxicology* 11, 323-336.

509 Houtman, C.J., Cenijn, P.H., Hamers, T., Lamoree, M.H., Legler, J., Murk, A.J., Brouwer, A.,  
510 2004. Toxicological profiling of sediments using *in vitro* bioassays, with emphasis on  
511 endocrine disruption. *Environmental Toxicology and Chemistry* 23, 32-40.

512 Houtman, C.J., Booij, P., Jover, E., del Rio, D.P., Swart, K., van Velzen, M., Vreuls, R.,  
513 Legler, J., Brouwer, A., Lamoree, M.H., 2006. Estrogenic and dioxin-like compounds in  
514 sediment from Zierikzee harbour identified with CALUX assay-directed fractionation  
515 combined with one and two dimensional gas chromatography analyses. *Chemosphere*, 65,  
516 2244-2252.

517 Jenkins, R.L., Wilson, E.M., Angus, R.A., Howell, W.M., Kirk, M., Moore, R., Nance, M.,  
518 Brown, A., 2004. Production of androgens by microbial transformation of progesterone in  
519 *vitro*: A model for androgen production in rivers receiving paper mill effluent.  
520 *Environmental Health Perspectives*, 112, 1508-1511.

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

525 Kavlock, R.J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L.E., Moore, J., Rolland, R.,  
526 Scott, G., Sheehan, D.M., Sinks, T., 1996. A report of the US EPA-sponsored workshop.  
527 *Environmental Health Perspectives* 107, 715-740.

528 Kinani, S., Bouchonnet, S., Bourcier, S., Creusot, N., Porcher, J.M., Ait-Aïssa, S., 2008a.  
529 Extraction and purification procedures for simultaneous quantification of phenolic  
530 xenoestrogens and steroid estrogens in river sediment by gas chromatography/ion trap  
531 mass spectrometry. *Rapid Communications in Mass Spectrometry* 22, 3651-3661.

- 532 Kinani, S., Bouchonnet, S., Bourcier, S., Porcher, J.-M., Aït-Aïssa, S., 2008b. Study of the  
533 chemical derivatization of zearalenone and its metabolites for gas chromatography-mass  
534 spectrometry analysis of environmental samples. *Journal of Chromatography A* 1190,  
535 307-315.
- 536 Labadie, P., Budzinski, H., 2005. Determination of steroidal hormone profiles along the Jalle  
537 d'Eysines River (near Bordeaux, France). *Environmental Science & Technology* 39,  
538 5113-5120.
- 539 Labadie, P., Hill, E.M., 2007. Analysis of estrogens in river sediments by liquid  
540 chromatography-electrospray ionisation mass spectrometry - Comparison of tandem mass  
541 spectrometry and time-of-flight mass spectrometry. *Journal of Chromatography A* 1141,  
542 174-181
- 543 Laville, N., Aït-Aïssa, S., Gomez, E., Casellas, C., Porcher, J.M., 2004. Effects of human  
544 pharmaceuticals on cytotoxicity, EROD activity and ROS production in fish hepatocytes.  
545 *Toxicology* 196, 41-55.
- 546 Laville, N., Balaguer, P., Brion, F., Hinfray, N., Casellas, C., Porcher, J.-M., Aït-Aïssa, S.,  
547 2006. Modulation of aromatase activity and mRNA by various selected pesticides in the  
548 human choriocarcinoma JEG-3 cell line. *Toxicology* 228, 98-108.
- 549 Lemaire, G., Mnif, W., Pascussi, J.M., Pillon, A., Rabenoelina, F., Fenet, H., Gomez, E.,  
550 Casellas, C., Nicolas, J.C., Cavailles, V., Duchesne, M.J., Balaguer, P., 2006.  
551 Identification of new human pregnane X receptor ligands among pesticides using a stable  
552 reporter cell system. *Toxicological Sciences* 91, 501-509.
- 553 Louiz, I., Kinani, S., Gouze, M.E., Ben-Attia, M., Menif, D., Bouchonnet, S., Porcher, J.-M.,  
554 Ben-Hassine, O.K., Aït-Aïssa, S., 2008. Monitoring of dioxin-like, estrogenic and anti-  
555 androgenic activities in sediments of the Bizerta lagoon (Tunisia) by means of in vitro

556 cell-based bioassays: contribution of low concentrations of polynuclear aromatic  
557 hydrocarbons (PAHs). *Science of the Total Environment* 402, 318-329.

558 Matejcek, D., Houserova, P., Kuban, V., 2007. Combined isolation and purification  
559 procedures prior to the high-performance liquid chromatographic-ion-trap tandem mass  
560 spectrometric determination of estrogens and their conjugates in river sediments. *Journal*  
561 *of Chromatography A*, 1171, 80-89.

562 Mnif, W., Pascussi, J.-M., Pillon, A., Escande, A., Bartegi, A., Nicolas, J.-C., Cavallès, V.,  
563 Duchesne, M.-J., Balaguer, P., 2007. Estrogens and antiestrogens activate hPXR.  
564 *Toxicology Letters* 170, 19-29.

565 Mossman, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to  
566 proliferation and cytotoxicity assays. *Journal of Immunology*, 65, 55-63.

567 Muller, M., Rabenoelina, F., Balaguer, P., Patureau, D., Lemenach, K., Budzinski, H.,  
568 Barcelo, D., De Alda, M.L., Kuster, M., Delgenes, J.P., Hernandez-Raquet, G., 2008.  
569 Chemical and biological analysis of endocrine-disrupting hormones and estrogenic  
570 activity in an advanced sewage treatment plant. *Environmental Toxicology and*  
571 *Chemistry* 27, 1649-1658.

572 Ohtake, F., Takeyama, K., Matsumoto, T., Kitagawa, H., Yamamoto, Y., Nohara, K.,  
573 Tohyama, C., Krust, A., Mimura, J., Chambon, P., Yanagisawa, J., Fujii-Kuriyama, Y.,  
574 Kato, S., 2003. Modulation of oestrogen receptor signalling by association with the  
575 activated dioxin receptor. *Nature* 423, 545-550.

576 Ohtake, F., Baba, A., Takada, I., Okada, M., Iwasaki, K., Miki, H., Takahashi, S.,  
577 Kouzmenko, A., Nohara, K., Chiba, T., Fujii-Kuriyama, Y., Kato, S., 2007. Dioxin  
578 receptor is a ligand-dependent E3 ubiquitin ligase. *Nature* 446, 562-566.

579 Ollivon, D., Garban, B., Blanchard, M., Teil, M.J., Carru, A.M., Chesterikoff, C., Chevreuil,  
580 M., 2002. Vertical distribution and fate of trace metals and persistent organic pollutants

581 in sediments of the Seine and Marne rivers (France). *Water Air and Soil Pollution* 134,  
582 57-79.

583 Pacyniak, E.K., Cheng, X.G., Cunningham, M.L., Crofton, K., Klaassen, C.D., Guo, G.L.,  
584 2007. The flame retardants, polybrominated diphenyl ethers, are pregnane X receptor  
585 activators. *Toxicological Sciences* 97, 94-102.

586 Pillon, A., Boussioux, A.M., Escande, A., Aït-Aïssa, S., Gomez, E., Fenet, H., Ruff, M.,  
587 Moras, D., Vignon, F., Duchesne, M.J., Casellas, C., Nicolas, J.C., Balaguer, P., 2005.  
588 Binding of estrogenic compounds to recombinant estrogen receptor alpha : Application to  
589 environmental analysis. *Environmental Health Perspectives*, 113, 278-284.

590 Reddy, S., Brownawell, B.J., 2005. Analysis of estrogens in sediment from a sewage-  
591 impacted urban estuary using high-performance liquid chromatography/time-of-flight  
592 mass spectrometry. *Environmental Toxicology and Chemistry* 24, 1041-1047.

593 Ryan, J.A., Hightower, L.E., 1994. Evaluation of heavy-metal ion toxicity in fish cells using a  
594 combined stress protein and cytotoxicity assay. *Environmental Toxicology and*  
595 *Chemistry* 13, 1231-1240.

596 Sanchez, W., Katsiadaki, I., Piccini, B., Ditche, J.M., Porcher, J.M., 2008. Biomarker  
597 responses in wild three-spined stickleback (*Gasterosteus aculeatus* L.) as a useful tool for  
598 freshwater biomonitoring: A multiparametric approach. *Environment International* 34,  
599 490-498.

600 Sanchez, W., Aït-Aïssa, S., Palluel, O., Ditche, J.M., Porcher, J.-M., 2007. Preliminary  
601 investigation of multi-biomarker responses in three-spined stickleback (*Gasterosteus*  
602 *aculeatus* L.) sampled in contaminated streams. *Ecotoxicology* 16, 279-287.

603 Soto, A.M., Calabro, J.M., Precht, N.V., Yau, A.Y., Orlando, E.F., Daxenberger, A., Kolok,  
604 A.S., Guillette, L.J., le Bizec, B., Lange, I.G., Sonnenschein, C., 2004. Androgenic and

- 605       estrogenic activity in water bodies receiving cattle feedlot effluent in eastern Nebraska,  
606       USA. *Environmental Health Perspectives* 112, 346-352.
- 607       Sumpter, J.P., 2005. Endocrine disrupters in the aquatic environment: An overview. *Acta*  
608       *Hydrochimica Et Hydrobiologica* 33, 9-16.
- 609       Thomas, K.V., Hurst, M.R., Matthiessen, P., McHugh, M., Smith, A., Waldock, M.J., 2002.  
610       An assessment of in vitro androgenic activity and the identification of environmental  
611       androgens in United Kingdom estuaries. *Environmental Toxicology and Chemistry* 21,  
612       1456-1461.
- 613       Urbatzka, R., van Cauwenberge, A., Maggioni, S., Vigano, L., Mandich, A., Benfenati, E.,  
614       Lutz, I., Kloas, W., 2007. Androgenic and antiandrogenic activities in water and sediment  
615       samples from the river Lambro, Italy, detected by yeast androgen screen and chemical  
616       analyses. *Chemosphere*, 67, 1080-1087.
- 617       Vethaak, A.D., Lahr, J., Schrap, S.M., Belfroid, A.C., Rijs, G.B.J., Gerritsen, A., de Boer, J.,  
618       Bulder, A.S., Grinwis, G.C.M., Kuiper, R.V., Legler, J., Murk, T.A.J., Peijnenburg, W.,  
619       Verhaar, H.J.M., de Voogt, P., 2005. An integrated assessment of estrogenic  
620       contamination and biological effects in the aquatic environment of The Netherlands.  
621       *Chemosphere* 59, 511-524.
- 622       Vindimian, E., Robault, C., Fillion, G., 1983. A method for co-operative and non co-operative  
623       binding studies using non-linear regression analysis on a microcomputer. *Journal of*  
624       *Applied Biochemistry*, 5, 261-268.
- 625       Vos, J.G., Dybing, E., Greim, H.A., Ladefoged, O., Lambre, C., Tarazona, J.V., Brandt, I.,  
626       Vethaak, A.D., 2000. Health effects of endocrine-disrupting chemicals on wildlife, with  
627       special reference to the European situation. *Critical Reviews in Toxicology* 30, 71-133.
- 628       Wilson, V.S., Bobseine, K., Lambright, C.R., Gray, L.E., 2002. A novel cell line, MDA-kb2,  
629       that stably expresses an androgen- and glucocorticoid-responsive reporter for the

630 detection of hormone receptor agonists and antagonists. *Toxicological Sciences* 66, 69-  
631 81.

## 632 **Figure legends**

633

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

637

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

641

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

645

646 Figure 4. (a) Androgenic and (b) anti-androgenic activities of the reference chemicals  
647 [dihydrotestosterone (DHT) and Flutamide (Flu)] and sediment extracts in MDA-kb2 cells.  
648 Results are expressed as percentage of the maximal luciferase activity induced by DHT at (a)  
649 10 nM and (b) 0.3 nM. Values are means of triplicates  $\pm$  SD.

650

651 Figure 5. (a) PXR-mediated dose-response curves of reference chemical (SR 12813) and  
652 sediment extracts (1: Rev, 2: Rho, 3: Lez, 4: VdV, 5: Ais) in HG<sub>5</sub>LN-PXR cells. Results are  
653 expressed as percentage of maximal luciferase activity induced by SR 12813 at 0.3  $\mu$ M. (b)  
654 Non specific effect of sediment extracts on constitutive luciferase expression in HG<sub>5</sub>LN cells.  
655 Results are expressed as percentage of luciferase activity in control cells. Values are means of  
656 triplicates  $\pm$  SD.

657

658

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 <sup>a</sup>	Low	Mixed	Urban dense	Urban dense	Mixed
Water quality <sup>b</sup>	Good	Very good	Bad	Very bad	Good
Biomarker responses in fish ( <i>Gasterosteus aculeatus</i> ) <sup>c</sup>	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

661  
 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

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

669  
 670  
 671

672 Table 3. Concentrations of the 16 PAHs and their relative contribution to the total dioxin-like  
 673 activity in sediment organic extracts from the five studied sites.  
 674

Chemicals	LOQ <sup>a</sup> (ng/g)	IEF <sup>b</sup>		Concentration in sampling sites in ng/g d.w.				
		BaP 4h	TCDD 24h	Ais	VdV	Rho	Rev	Lez
Nap	0.11	n.i. <sup>c</sup>	n.i.	n.d. <sup>d</sup>	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
<i>Sum-PAHs (ng/g d.w.)</i>				<i>209.3</i>	<i>1462.8</i>	<i>236.5</i>	<i>11976.1</i>	<i>23077.7</i>
Chem-BaP-EQ (ng/g d.w.)				81	550	1978	8740	15037
Bio-BaP-EQ (ng/g d.w.)				200	910	7334	31556	75435
<i>Chem-BaP-EQ / Bio-BaP-EQ (%)</i>				<i>41</i>	<i>60</i>	<i>27</i>	<i>28</i>	<i>20</i>
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
<i>Chem-TCDD-EQ / Bio-TCDD-EQ (%)</i>				<i>16</i>	<i>16</i>	<i>40</i>	<i>27</i>	<i>37</i>

675  
 676 <sup>a</sup> LOQ: limits of quantification provided by the GC-MS method, <sup>b</sup> IEF: induction equivalent factors relative to  
 677 BaP after 4 h and to TCDD after 24 h of exposure (from Louiz et al., 2008), <sup>c</sup> n.i.: no EROD induction detected  
 678 within the 0.1 nM to 10 µM concentration range, <sup>d</sup> n.d.: not detected or below quantification limit.  
 679

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 <sup>a</sup>	LOQ <sup>b</sup> (ng/g)	Concentration in sediment extracts (ng/g d.w.)				
				Ais	VdV	Rho	Rev	Lez
<i>Natural estrogens</i>	$\alpha$ -E2	0.02	0.04	0.04	n.d.	0.2	n.d.	n.d.
	$\beta$ -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.
<i>Synthetic estrogens</i>	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.
<i>Alkylphenols</i>	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
<i>Parabens</i>	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.
<i>PAHs metabolites</i>	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
<i>Pesticides</i>	Endosulfan( $\alpha$ )	2.0E-06	0.21	n.d.	n.d.	n.d.	63.84	n.d.
	Endosulfan( $\beta$ )	2.0E-06	1.25	n.d.	n.d.	n.d.	29.2	n.d.
	<i>o,p'</i> -DDT	1.7E-05	0.22	6.52	n.d.	n.d.	7.25	n.d.
<i>Phytoestrogen</i>	Equol	2.8E-04	0.15	n.d.	n.d.	0.17	n.d.	n.d.
<i>Mycoestrogens</i>	$\alpha$ -ZAL	0.14	0.13	n.d.	n.d.	n.d.	< LOQ	n.d.
	$\beta$ -ZAL	0.03	0.02	n.d.	n.d.	n.d.	< LOQ	n.d.
Chem-E2-EQ (ng/g) <sup>d</sup>			-	0.28	0.79	0.59	1.82	0.19
Bio-E2-EQ (ng/g) <sup>e</sup>			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 <sup>a</sup> EEF: estradiol equivalence factors relative to estradiol, determined as described in the Materials and Methods  
 686 section, <sup>b</sup> LOQ: limit of quantification, <sup>c</sup> n.d.: not detected or below quantification limits, <sup>d</sup> Chem-E2-EQs:  
 687 chemical estradiol equivalents, <sup>e</sup> Biol-E2-EQs: biological estradiol equivalents based on EC<sub>25</sub> 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 <sup>a</sup>	SREF <sup>a</sup>	LOQ <sup>b</sup> (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( $\alpha$ )	0.058	0.029	0.21	n.d.	n.d.	n.d.	63.84	n.d.
<i>o,p'</i> -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.
$\beta$ -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) <sup>d</sup>			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) <sup>d</sup>				0.34	0.47	0.39	4.26	0.10
Bio-SR12813-EQ (ng/g) <sup>d</sup>			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 <sup>a</sup> 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. <sup>b</sup> LOQ: limit of quantification. n.a.: non active compound, n.d. not  
 698 detected.  
 699