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2 **sediments from reference and impacted small rivers**

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17

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

3

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

2. Materials and methods

91

92

2.1. Chemicals and reagents

93

94 List, abbreviation and source of analytical standards are given in Table 2. Flutamide (Flut), 3-

95 (4,5-dimethylthiazol-2-ol)-2,5-diphenyltetrasodium bromide tetrazolium (MTT), 5α-

96 dihydrotestosterone (DHT), as well as the analytical internal standards including $[^{13}C_6]$4-n-

97 nonylphenol, $[^2H_2]$17β-estradiol, $[^2H_{10}]$phenanthrene, $[^2H_{10}]$acenaphthene, $[^2H_{12}]$perylene and

98 $[^2H_{12}]$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 β-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

2.2. Site description and sampling

108

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_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

161   MELN, HG_5LN-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_2 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 µm) from Varian. All experiments were performed by automatically injecting

194   2.0 µL of sample in the splitless mode at a rate of 50 µL/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 to300 °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, γ-HCH, vinclozolin, metolachlor, α-

203   endosulfan, *o,p'*-DDT, β-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 µA. 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; desolvatation temperature, 450°C. Nitrogen was used as both the

222 nebulizing (50 L/h) and desolvatation 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^{TM} 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   Chem-ref-EQ $= \sum (C_i \times$ ref-EF$_i$), where, for a given chemical i, C_i is the measured

244   concentration in a sample and 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: ref-EF$_i = $ EC$_{25}$ of reference compound / EC$_{25}$ of

247   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***

11

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_{50} 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 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.

334

335

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 $\beta E2$ 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; 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

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

19

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.

471

478

479   **5. References**

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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) 17β-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 β-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$_5$LN-PXR cells. Results are

653 expressed as percentage of maximal luciferase activity induced by SR 12813 at 0.3 μM. (b)

654 Non specific effect of sediment extracts on constitutive luciferase expression in HG$_5$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[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

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

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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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 [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*

675
676 [a] LOQ: limits of quantification provided by the GC-MS method, [b] IEF: induction equivalent factors relative to
677 BaP after 4 h and to TCDD after 24 h of exposure (from Louiz et al., 2008), [c] n.i.: no EROD induction detected
678 within the 0.1 nM to 10 μM concentration range, [d] 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[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

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

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

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