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Monitoring of dioxin-like, estrogenic and anti-androgenic activities in sediments of the Bizerta lagoon (Tunisia) by means of *in vitro* cell-based bioassays: contribution of low concentrations of polynuclear aromatic hydrocarbons (PAHs)

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24 ***Abstract***

25 We used an array of *in vitro* cell-based bioassays to assess dioxin-like, estrogenic and
26 (anti-)androgenic activities in organic extracts of sediments from the Bizerta lagoon,
27 one of the largest Tunisian lagoons subjected to various anthropogenic and industrial
28 pressures. The sediments were sampled both in winter and summer 2006 in 6 stations
29 differently impacted and in one reference station located in the seawards entrance of
30 Ghar el Mehl lagoon. Chemical analyses of the 16 priority PAHs showed that the
31 sediments were low to moderately contaminated (2-537 ng/g dry weight). By using the
32 estrogen- (MELN) and androgen-responsive (MDA-kb2) reporter cell lines, significant
33 estrogenic and anti-androgenic activities were detected only in the Menzel Bourguiba
34 (MB) site, the most contaminated site, both in winter and summer. By using 7-
35 ethoxyresorufin-O-deethylase (EROD) induction in the fish PLHC-1 cell line after both
36 4 and 24 h of cell exposure, dioxin-like activities were detected in all analysed samples.
37 Dioxin-like activities were higher after 4 h exposure, and varied according to the sites
38 and the sampling season. While highly significant correlation was observed between
39 bioassay- and chemical analyses-derived toxic equivalents (TEQs), PAHs accounted for
40 only a small part (up to 4 %) of the detected biological activities, suggesting that other
41 readily metabolised EROD inducing compounds were present. This study argues for the
42 use of short time exposure to assess biological TEQs in low contaminated samples and
43 provides new induction equivalent factors (IEF_{4h}) for 16 PAHs in the PLHC-1 cell line.
44 Finally, our results stress the need to further characterise the nature of organic chemical
45 contamination as well as its long-term impacts on aquatic wildlife in the Bizerta lagoon.
46

47 Keywords: Bizerta lagoon sediments, *in vitro* cell bioassays, estrogenicity, anti-
48 androgenicity, dioxin-like activity, PAH contamination, benzo[*a*]pyrene- and dioxin-
49 equivalents.
50

51 **1. Introduction**

52 Bizerta lagoon, the second largest lagoon in Tunisia, is located in an economically very
53 important area in northern Tunisia (Fig.1). This lagoon is submitted to many anthropic
54 pressures including urbanisation, industrial activities (cement works, metallurgical
55 industry, boatyard, tyre production factories...), as well as naval and commercial
56 shipping harbours. Lagoon shores have also been used as open-air waste-dumping sites.
57 The direct and indirect discharges of urban and industrial wastes and runoff lead to the
58 chemical contamination of the lagoon by various toxic compounds such as organo-
59 chlorinated pesticides (Cheikh et al., 2002), halogenated aromatics compounds like
60 polychlorobiphenyls (PCBs) (Derouiche et al., 2004), polycyclic aromatic hydrocarbons
61 (PAHs) (Trabelsi and Driss, 2005), heavy metals (Yoshida et al., 2002) and organotins
62 (Mzoughi et al., 2005). The presence of such potentially toxic compounds in this
63 aquatic ecosystem has led scientists to investigate biological impacts on aquatic
64 organisms. Recently, some biological alterations have been reported, like imposex
65 incidence in the muricid gastropod *Hexaplex trunculus* (Lahbib et al., 2007), inhibition
66 of acetylcholinesterase activity in clams and mussels sampled (Dellali et al., 2001) or
67 the oxidative damage to DNA in clam gills (Jebali et al., 2007). Fish are also possibly
68 affected by lagoon pollution. Indeed, substantial vertebral deformities were recently
69 reported in three Gobiidae species, sedentary and benthic fish sampled in different sites
70 of the Bizerta lagoon (Louiz et al., 2007). Interestingly, those morphological alterations
71 in adults were found to be correlated with the degree of sediment contamination by
72 PAHs, suggesting that in situ long-term chemical exposure at these sites could be
73 responsible for integrated biological effects related to essential physiological functions,
74 like development or reproduction (Louiz et al., 2007). However, these data remain
75 scarce and much information is still needed in order to characterise the toxic potency
76 and the identity of biologically active chemicals that are present in this lagoon.

77 In this context, the present study was undertaken in order to provide a first evaluation of
78 the presence of bioactive organic contaminants, namely dioxin-like and endocrine
79 disrupting chemicals, using *in vitro* bioassays. It is now recognised that *in vitro*
80 mechanism-based cellular bioassays serve as valuable bio-analytical tools for the
81 detection and quantification of biologically active chemicals in environmental mixtures
82 such as river sediment, surface waters or aqueous effluents (Eggen and Segner, 2003).

83 The principle of such assays relies on a common mode of action of chemicals, by which
84 they initiate their toxicity, like their ability to bind to nuclear receptors or transcription
85 factors and to subsequently activate or inhibit target genes. A very relevant group of
86 contaminants are the so-called dioxin-like compounds, which include dioxins, furans,
87 planar polychlorobiphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), etc.
88 These chemicals bind to the aryl hydrocarbon receptor (AhR) and thereby activate the
89 transcription of several genes, including those encoding for xenobiotic or hormone
90 metabolising enzymes, such as CYP1A(Denison and Nagy, 2003). The AhR plays
91 major roles in the mediation of developmental and reproductive toxicity of dioxin-like
92 compounds (Hahn, 2002).
93 Apart from dioxin-like compounds, a number of environmental chemicals can act as
94 endocrine disrupters (EDs) by altering the normal functioning, synthesis and/or
95 metabolism of endogenous hormones, and thereby affect growth, development and
96 reproduction in wildlife and humans (Kavlock et al., 1996). One major molecular
97 mechanism of action of EDs involves their ability to bind to steroid hormone receptors,
98 i.e. estrogen (ER) or androgen (AR) receptors, and to subsequently modulate the
99 expression of target genes responsible for hormonal cellular response. In the aquatic
100 environment, a wide range of chemicals (e.g. natural and synthetic hormones,
101 phytoestrogens, pesticides, alkylphenols, bisphenol A, phtalates, and so on) have been
102 identified as estrogenic, anti-androgenic or androgenic compounds through their direct
103 binding to the ER or AR (Kelce et al., 1995; Sohoni and Sumpter, 1998; Sonnenschein
104 and Soto, 1998). In addition, environmental AhR ligands can also interact with the ER
105 signalling pathway through a cross-talk between activated AhR and ER, leading to
106 modulation of ER-regulated gene expression (Ohtake et al., 2003; Navas and Segner,
107 2001). Upon field exposure, EDs have been shown to adversely affect reproduction
108 capabilities in aquatic wildlife (Tyler et al., 1998; Jobling et al., 2002).
109 The aim of this study was to assess the presence of compounds able to bind to the
110 estrogen (ER), androgen (AR) or aryl hydrocarbon receptors (AhR) in organic extracts
111 of Bizerta lagoon sediments, by using *in vitro* assays. The estrogenic and androgenic
112 activities in the extracts were assessed by using established human reporter MELN
113 (Balaguer et al., 1999) and MDA-kb2 (Wilson et al., 2002) cell lines that stably express
114 the luciferase reporter gene under the control of the human ER or AR, respectively. The

115 AhR-mediated activities were detected in the hepatoma fish PLHC-1 cell line (Ryan and
116 Hightower, 1994), by assessing CYP1A enzymatic activity. Because dioxin-like
117 activities were found to be predominant in these sites, chemical analyses of target PAHs
118 were performed in parallel in order to evaluate their contribution in measured biological
119 activities.
120
121
122

123 ## 2. Materials and methods

124 ### *2.1. Chemicals*

125 17β-estradiol (E_2), 5α-dihydrotestosterone (DHT), as well as the 16 standard PAHs
126 (listed in Table 2) were purchased from Sigma-Aldrich (St Quentin Fallavier, France).
127 ICI 182,780 (ICI) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were obtained from
128 Tocris Bioscience (Ellisville, USA) and Promochem (Molsheim, France), respectively.
129 All standards were of 98.1-99.8% purity. For bioassay experiments, stock solutions (10
130 mM) were prepared in either dimethylsulfoxide (DMSO) or methanol and stored at -
131 20°C. For chemical analyses of PAHs, stock solutions of each compound were prepared
132 at a concentration of 1 $mg.mL^{-1}$ in toluene and stored at 4°C. Standard solutions were
133 mixed to provide a solution of 10 $\mu g.mL^{-1}$ of each PAH. A 1000 $\mu g.mL^{-1}$ mixture
134 solution of the following deuterated PAHs: [$^2H_{10}$]phenanthrene (PA-d10),
135 [$^2H_{10}$]acenaphthene (Acp-d10), [$^2H_{12}$]perylene (P-d12) and [$^2H_{12}$]chrysene (CHR-d12)
136 (St Quentin Fallavier, France), was used as the internal standard for quantification.

137 ### *2.2. Studied sites and sample collection*

138 - *Study area*

139 The sediment sampling sites were located in Bizerta and Ghar el Melh lagoons (Fig.1).
140 The Bizerta lagoon is about 150 km^2 and is linked to the Mediterranean Sea and Lake
141 Ichkeul by straight channels. The exchange of water with the Mediterranean Sea
142 determine the salinity of the lagoon, which varies between 32.5‰ and 38.5‰. The
143 water temperature ranged from 13°C to 32.5°C in 2006 in winter and summer,
144 respectively. A relatively undisturbed site (Mahmoudi, 2003) located at the seawards
145 entrance of Ghar el Melh lagoon (Fig. 1) was chosen as a reference station.

146 - *Sediment sampling and extraction*

147 Surface sediments were collected in January 2006 and July 2006 at six stations in
148 Bizerta lagoon and one station in the Ghar el Melh lagoon. These stations were chosen
149 as representative of the different anthropic pressures present in the study area (Table 1):
150 CA, NJ, MB, MJ and ML were considered as impacted areas, while MR and GH were
151 considered as low impacted or reference sites. The upper 10 cm-surface layer of
152 sediment (about 500 g) were sampled, homogenised and stored in methanol-rinsed glass
153 bottles at -20°C until processing. Before extraction, freeze dried sediments were sieved
154 using a 2-mm sieve. Five grams of dry sediment were then extracted by a
155 heptane:acetone (1:1) mixture using Accelerated Solvent Extraction (ASE 200; Dionex,
156 France). ASE extraction was performed at 90°C, 103 Bars. Sediment extracts were
157 completely evaporated under a gentle nitrogen stream and dissolved in 1 ml of
158 methanol. The final methanol extracts were then subjected to chemical and bioassays
159 analyses, as described below.

160 ***2.3. Chemical analyses***

161 All PAH analyses were carried out on a Varian "CP3800" gas chromatograph system
162 equipped with a "CP8400" autosampler and coupled to a Saturn "2000" ion trap mass
163 spectrometer (Varian, Les Ulis, France). The chromatographic separation was
164 performed on a 60 m "Factor Four VF-10-MS" capillary column (internal diameter:
165 0.25 mm, film thickness: 0.25 µm) from Varian. All experiments were performed by
166 automatically injecting 1.5 µL of sample in the splitless mode at a rate of 50 $\mu L.s^{-1}$.
167 Helium (purity: 99.999%) was used as carrier gas at a constant flow of 1.0 $mL.min^{-1}$.
168 The injector temperature was set to 280 °C. The split valve opened after 1.5 min, with a
169 split ratio of 35/100. The capillary column was increased from an initial temperature of
170 70°C, held for 0.5 min, increased at 10°C/min up to 280°C where it was held for 41.50
171 min. The total duration of GC analysis was 63.00 min. The manifold, ion trap source
172 and transfer line temperatures were set to 120, 220 and 300°C, respectively. Ions were
173 formed under electro-ionization at 70 eV with an emission current of 20 µA. The
174 electron multiplier voltage was set to 1550 V. Spectra were recorded using the
175 automatic gain controller (AGC) function with a target value of 20000.

176 The mass spectrometer was operated in the "Selected Ion Storage" (SIS) mode.
177 Molecular ions $[M]^{+\cdot}$ as well as $[M\text{-}H]^{+}$ and $[M(^{13}C)]^{+\cdot}$ ions were stored and monitored
178 for each compound. The m/z ratios of the quantification/confirmation ions for all PAHs

179 were chosen according to the 96/23/CE directive published in the Journal of the
180 European Communities and notified under the number C(2002)3044. Calibration curves
181 were plotted using 10, 50, 100, 300, 500, 700, and 1000 $ng.mL^{-1}$ standard solutions.
182 Quantitative data were determined by internal standard calibration. The response was
183 linear (r^2 greater than 0.995) on the whole concentration range for each of the
184 considered PAHs. Quantification limits (LOQ, estimated for a signal-to-noise ratio of
185 10) ranged from 0.02 to 1.20 $ng.g^{-1}$ according to the PAH considered (Table 2).

186 ***2.4. In vitro cellular bioassays***

187 *- Estrogenic and (anti-)androgenic activities in reporter gene assays*

188 The estrogenic and (anti-)androgenic activities of the extracts were monitored by using
189 the MELN and MDA-kb2 cell lines, respectively. The MELN cell line was obtained
190 from Patrick Balaguer (INSERM, Montpellier, France). These cells were obtained by
191 stable transfection of MCF-7 cells by an ERE-βGlob-Luc-SVNeo plasmid and selected
192 with 1 $mg.ml^{-1}$ G418 (Balaguer et al., 1999). They were routinely cultured in phenol red
193 containing Dulbecco's Modified Eagles's Medium (DMEM), supplemented with 5%
194 foetal calf serum (FCS), 1% non essential amino acids and penicillin/streptomycin (50
195 U/ml each) in a 5% CO_2 humidified atmosphere at 37°C. For experiments, MELN cells
196 were left to incubate for two days in phenol red free DMEM supplemented with 3%
197 dextran- charcoal coated FCS (DCC medium) before seeding in white opaque 96-wells
198 culture plates. After another 24 h period, the cells were exposed for 16 hours in
199 triplicates to vehicle (solvent control, 0.5% v/v), estradiol (E2; positive control), blank
200 extraction procedure or serial dilutions of sediment organic extracts. The solvent
201 concentration in the culture medium was always 0.5% v/v. At this concentration, it does
202 not affect either cell viability or luciferase activity. After exposure, the medium was
203 replaced by 50 µl of DCC-medium containing 3.10^4 M D-luciferin (Sigma) and the
204 luminescence signal in living cells was read after 5 minutes with a microtiter plate
205 luminometer (µBeta, Wallac). The MDA-kb2 cell line (ATCC, CRL-2713) was derived
206 from MDA-MB-453 cells. They were stably transfected by a MMTV promoter-
207 luciferase plasmid construct, which is under the control of endogenous androgen (AR)
208 and glucocorticoid receptors (GR) (Wilson et al., 2002). These cells were routinely
209 grown at 37°C under humidified air atmosphere in L15 Medium (Sigma) supplemented
210 with 10% foetal calf serum (FCS), 1% (v/v) non essential amino acids and

211 penicillin/streptomycin (50 U/ml each). For sample analyses, cells were exposed to
212 serial dilutions of extracts either in the absence (for agonist activity) or in the presence
213 of 0.3 nM of DHT in the culture medium (for AR antagonist activity). Exposure to
214 reference chemicals or sample extracts was carried out for 16 hours in 96-well white
215 opaque plates (5 x 10^4 cells per well) in complete L15 medium, and luciferase activity
216 was then monitored exactly as described with MELN cells. In both assays, results are
217 expressed as percent of maximal luciferase activity induced by the positive controls,
218 respectively E2 10 nM or DHT 1 nM in MELN or MDA-kb2.

219 *- 7-Ethoxyresorufin-O-deethylase (EROD) activity assay in PLHC-1 cell line*

220 The PLHC-1 cells (ATCC, CRL-2406) were routinely grown at 30°C in E-MEM culture
221 media supplemented with 10% foetal calf serum and 1% antibiotics in a 5% CO_2
222 humidified atmosphere. For experiments, cells were seeded in 96-well plates at a rate of
223 5 x 10^4 cells per well. After 24 hours of incubation, cells were exposed to test chemicals
224 or sample extracts for either 4 h or 24 h and then processed for EROD activity in intact
225 cells, exactly as previously described (Laville et al., 2004). Results were expressed as
226 percent of EROD activity induced by the positive control (TCDD 1 nM).

227 *- Cell viability*

228 Cellular viability after sample exposure of the different cell lines was evaluated by
229 using the methyl-thiazol-tetrazolium (MTT) assay (Mosmann, 1983), as previously
230 described (Laville et al., 2004).

231 ***2.5. Data analyses***

232 In all assays, each sample was tested at various concentrations in at least two
233 independent experiments, which always included both negative (solvent) controls and
234 complete dose-response for reference chemical (TCDD, BaP, E2 or DHT).
235 Experimental data were expressed as means ± standard deviation (SD). Statistical
236 significance ($p < 0.05$) of treatment effects was tested by using Student *t*-test. Pearson's
237 linear regression was used for correlation analyses. The SPSS™ software version 10.1
238 was used for the statistical analysis.
239 Dose-response curves were modelled by using the Regtox 7.5 Microsoft Excel™ macro
240 (Vindimian et al. 1983). This macro uses the Hill equation model and allows calculation
241 of EC_{50} (concentrations of test chemical or sediment extract that induce 50% of
242 maximal response). Bioassay-derived TCDD- (TCDD-EQ), benzo[*a*]pyrene- (BaP-EQ)

243 or E2-equivalents (E2-EQ) were determined by dividing the EC_{50} of the reference
244 chemical (expressed in ng/L) by that of the sample (expressed as equivalent gram of dry
245 sediment per litre). Instrumentally derived toxic-equivalents (TEQs) for PAHs were
246 determined according to the following equation: TEQcal = $\Sigma([PAH_i]\cdot IEF_i)$, where,
247 $[PAH_i]$ is the measured concentration in the sample and IEF_i is the induction equivalent
248 factor, for a given compound (*i*). IEFs were determined as the ratio of EC_{50} as mass
249 concentration of reference compound (TCDD or BaP) to that of compound *i*. In the
250 present study, because no IEF data after short term exposure to PAHs were found in the
251 literature for the PLHC-1 cell line, IEFs for all 16 PAHs were determined in PLHC-1
252 cells after both 4 and 24 h of exposure to individual PAHs (see results section).
253
254 **3. Results**

3. Results

255 ***3.1. Responses of in vitro assays to reference ER, AR and AhR ligands***
256 The sensitivity of the three cellular assays in our assay conditions was first evaluated by
257 using reference chemicals (Fig. 2). In our experiments, the EC_{50s} of E2 in MELN cells
258 and DHT in MDA-kb2 cells were 0.03±0.01 and 0.145±0.065 nM (mean ± SD of three
259 independent experiments), respectively (Fig 2a). In PLHC-1 cells, patterns of EROD
260 induction by BaP and TCDD varied according to the exposure duration (Fig 2b). BaP
261 was more potent after 4 h (EC_{50} 4.1±3.3 nM) than after 24 h (EC_{50} 380±140 nM),
262 whereas TCDD was almost equipotent at both duration of exposure (EC_{50} 011±0.01 nM
263 at 4 h, 0.09±0.001 nM at 24 h). Maximal EROD activities induced by TCDD 1 nM were
264 about 30 and 160 pmol resorufin/mg protein/min after 4 and 24 h of exposure,
265 respectively. Overall, the response induced by reference ligands, were in line with the
266 reported sensitivity of these cell lines, which validates their use in the present study.
267 ***3.2. Estrogenic and anti-androgenic activities in sediments extracts***
268 The effects of sediment extracts on ER, AR and cytotoxicity assays were limited. At the
269 highest tested concentration (i.e. 0.5 % v/v of extract), none of the extracts exerted
270 significant alteration of cellular viability as assessed by the MTT assay in the three cell
271 lines (data not shown). Among all samples, significant estrogenic and anti-androgenic
272 activities were detected only in the MB site, both in summer and winter samples (Fig.
273 3). In MELN cells, these activities were observed at the same sample concentration for
274 the two seasons and corresponded to 0.22 and 0.29 ng E2-Eq/g in winter and summer,

275 respectively. All other samples were below the detection limit (i.e. 0.011 ng E2-Eq/g).
276 Co-exposure with the pure antiestrogen ICI 182,780 led to a complete inhibition of the
277 estrogenic activity of MB extracts (result not shown), thus indicating that the detected
278 activity involved an ER-dependent mechanism in MELN cells. Interestingly, MB
279 sediment extracts also exerted anti-androgenic activity in MDA-kb2 cells, although
280 these activities were weak (20 % decrease of luciferase induced by DHT 0.1 nM). In
281 addition, no androgenic or anti-estrogenic activities were detected in any of the analysed
282 samples (data not shown).

283 ***3.3. In vitro dioxin-like activities in sediment extracts***

284 The induction of EROD in PLHC-1 cells by sediment extracts was assessed after both 4
285 and 24 hours of exposure in order to distinguish between HAP-like compounds that are
286 rapidly biotransformed in the cells and dioxin-like chemicals that are persistent in the
287 cells after 24 h. As shown in figure 4, all extracts were able to induce EROD activity
288 that depended on concentration and exposure duration, and with varying potencies
289 between the sites studied.

290 First, the time-course study showed that exposure duration affected both the magnitude
291 of EROD induction and EC_{50} values. Indeed, higher activities were observed after 4
292 hours of cell exposure (Fig. 4a,b) than after 24 hours (Fig. 4c,d). This pattern of EROD
293 responses suggests that the chemicals responsible for activation are rapidly metabolised
294 into the cells, which supports the hypothesis for a major role of PAH-like chemicals in
295 the observed EROD induction. Second, sample EC_{50s} varied by up to two orders of
296 magnitude between the different sites (Fig 4a, b). The extracts from MB site, which is
297 subjected to human industrial and urban activity nearby (Table 1), were the most active
298 samples with equal potency in winter and summer. On the contrary, the reference site
299 GH, was about 15 and 360 times less active than MB in winter and summer,
300 respectively. Regarding seasonal variation, it is noteworthy that, although similar site
301 ranking was observed between summer and winter, higher activities were observed in
302 winter in all stations, except for MB site, which was still highly potent in summer.

303 ***3.4. Concentrations of PAHs in sediment extracts***

304 On the basis of the results provided by the *in vitro* PLHC-1 assay at various exposure
305 duration, we suspected a significant contribution of PAHs to the biological responses.
306 To test this hypothesis, we performed analyses of the 16 US EPA priority PAHs in the

307 organic extracts. Overall, the results given in Table 2 showed that sediments presented
308 low to moderate PAH contamination. The concentrations of analysed PAHs ranged
309 from 1 ng/g in the GH reference site up to 537 ng/g in MB site, identified as the most
310 responsive site by *in vitro* assays. As observed with the EROD induction bioassays,
311 some seasonal variations were also observed, the total PAH content being substantially
312 higher in winter than in summer especially in sites CA, ML and MJ, but with the
313 exception of the MB site which had higher PAH content in summer.

314 ***3.5. Determination and comparison of biological and chemical-derived TEQs in***
315 ***sediment extracts***

316 In order to allow a quantitative comparison between chemical and biological data, we
317 applied the "toxic equivalent" (TEQ) approach to our data set. To derive chemical
318 analyses-based TEQ, we first determined EROD induction equivalent factors (IEF) of
319 individual PAHs at both times of exposure in our assay conditions. To this end, we
320 exposed PLHC-1 cells to various concentrations of individual PAHs (0.1 nM to 10 µM)
321 for 4 h and 24 h and determined their EC_{50}. The results, as per Table 3, showed that, on
322 the basis of EC_{50} values, all tested PAHs were more active after 4 h than after 24 h of
323 exposure. Such differences were also noticed when comparing IEFs relative to TCDD at
324 both times of exposure (Table 3). For active PAHs, the EC50s were found 5-fold
325 (Pyrene) up to 100-fold (Benz[*a*]Anthracene) more sensitive at 4 h as compared to 24 h.
326 Furthermore, fluorene and acenaphtylene were found to induce EROD activity after 4 h,
327 while they were inactive after 24 h within the range of tested concentrations, as
328 previously described in this cell line (Fent and Bätscher, 2000). Fluoranthene,
329 phenanthrene, benzo[*ghi*]perylene, acenaphtene, and anthracene showed no significant
330 response at both exposure durations.

331 The IEFs derived from these experiments were then used to derive toxic-equivalent
332 values from chemical analyses in sediments extracts. BaP-EQs and TCDD-EQs were
333 calculated by using $IEF\text{-}_{BaP\ 4h}$ and $IEF\text{-}_{TCDD\ 24h}$ given in Table 3, respectively. The
334 results presented in Table 4 showed very good concordance between the ranking of sites
335 given by biological- and instrumental-derived BEQs and TEQs. Furthermore, the
336 regression analyses (Fig. 5) showed highly significant positive correlation between
337 BEQchem and BEQbiol (R^2=0.95, $p<0.001$) and, to a fewer extent, between TEQchem
338 and TEQbiol (R^2=0.90, $p<0.01$). However, when comparing toxic-equivalent values in

339 Table 4, the BEQchem and TEQchem values explained only a small part (0.6 to 3.7%)
340 of the biological activities detected by the bioassay, suggesting that other EROD
341 inducing compounds than the 16 analysed PAHs are present in the extracts.
342

343 4. Discussion

344 By means of an array of *in vitro* bioassays, this study examined for the first time the
345 possible occurrence of ER-, AR- and AhR-mediated activities in sediments of the
346 Bizerta lagoon. The main outcomes were a low contamination of this lagoon by
347 estrogen-like or antiandrogen-like compounds while dioxin-like compounds were
348 detected in all analysed samples. PAHs, but also other unidentified chemicals,
349 contributed significantly to the dioxin-like activities detected by the bioassays.
350 Overall, the dioxin-like activities and chemical analyses showed large variations
351 between the different sites, which could be related to the industrial and urban activities
352 in the site areas. The site of Menzel Bourguiba (MB) provided the most active samples,
353 followed to a lesser extent by the channel (CA) and Menzel Abderrahmen (ML). These
354 sites are located in areas with intensive urban and industrial activities (Table 1)
355 characterised by metallurgical industry and ship building (MB), solid waste landfills
356 (MB, CA, ML), cement factories and commercial harbours (CA). Oppositely, the GH
357 and MR sites, located in non-urbanised and non-industrial zones, were not exposed to
358 any identified direct sources of pollution. Both sites had the lowest dioxin-like and total
359 PAH content in our study. In addition, the site ranking given by our data correlates with
360 previous studies that compared sediment contamination by PAHs (Trabelsi and Driss,
361 2005) or PCBs (Derouiche et al., 2001) in these sites among others of the Bizerta
362 lagoon.
363 With regard to total PAH concentrations, Bizerta lagoon sediments can be considered as
364 low to moderately polluted as compared to other marine ecosystems. In industrialized
365 coastal areas, much higher PAH concentrations, *i.e.* up to several µg/g sediment, can be
366 found, although the concentration ranges usually vary between neighbour sites
367 depending on the presence or not of a local source of pollution. For instance, reported
368 PAH concentrations in marine sediments ranged from 626 to 3766 ng/g in the Mersey
369 Estuary, U.K. *(*Vane et al., 2007), 1 to 20,440 ng/g in French Mediterranean coasts
370 (Baumard et al., 1998), or 7 to 640 ng/g in the Black Sea (Readman et al., 2002). In the

371 Bizerta lagoon**,** Trabelsi and Driss (2005) recorded slightly greater PAH concentrations
372 (83-450 ng/g) in sediments sampled in 2001 than in our study. Interestingly, in their
373 study, the MB site was found to be the most contaminated one with a total PAH content
374 of 450 ng/g (Trabelsi et Driss, 2005), which is close to our data. Globally, the levels of
375 concentrations of PAHs can be considered as relatively low considering the industrial
376 activities and urbanization that have been developed in the vicinity of the Bizerta
377 lagoon.
378 Seasonal variations were also observed when comparing winter and summer surveys, as
379 shown by both *in vitro* dioxin-like activities and PAH concentrations that were lower in
380 summer. In the same lagoon, Mzoughi et al. (2002) also reported similar seasonal
381 differences with higher PAH concentration in winter samples than in summer. Such
382 variations probably reflect a higher abiotic and biological degradation of organic
383 chemicals in the hot season. In addition, several PAH degrading bacterial strains were
384 recently isolated from Bizerta lagoon sediments (Ben Said et al., 2007), which
385 demonstrated the high PAH biodegradation capabilities of sediment microflora in this
386 lagoon.
387 In the PLHC-1 assay, higher AhR-mediated activities observed after 4 h of exposure
388 than after 24 h (Fig.4) led us to question whether or not rapidly metabolised active
389 compounds, like PAHs, were responsible for EROD induction. Indeed, the B[a]P-EQs
390 and TCDD-EQs derived from the PLHC-1 bioassay were positively correlated to toxic-
391 equivalents derived from PAH analyses (Table 4, Fig. 5). Similar significant correlation
392 has been reported (Michallet-Ferrier et al., 2004, Vondracek et al., 2001) between
393 bioassay and instrumentally derived TEQ in river sediments that have been highly
394 contaminated by PAHs . Our study shows that such a relationship is also demonstrated
395 for samples with low levels of contamination by the use of different duration of
396 exposure (e.g. 4 and 24 h). However, comparison of the amounts of toxic-equivalents
397 given by both methodologies (Table 4) indicated that only a small part of the B[a]P-EQs
398 (up to 3%) was accounted for by the 16 PAHs measured in the samples.
399 It is however noted that some caution should be observed when estimating bioassay-
400 derived TEQ values in complex mixtures because of non-parallel dose-response curves
401 or variation in maximal levels of EROD induction, a common feature with
402 environmental samples (Villeneuve et al., 2000). In our study, varying maximal EROD

403 induction levels were observed between samples (Fig. 4), and the TEQ values were
404 derived from median activities (defined as EC_{50}) of the samples, as given by non linear
405 regression of the data. This calculation mode was chosen in order to process all sample
406 data in the same way and to allow inter-site comparison. In addition, in order to estimate
407 a possible bias due to uncertainty in calculation, we determined TEQs on the basis of
408 EC_{20} ratios and found that EC_{20}-based TEQs were lower, but only slightly (by less than
409 two fold), than EC_{50}-based TEQs (data not shown). Hence, although bioassay-derived
410 TEQs may be considered as semi-quantitative data, we assume that uncertainty in their
411 determination does not explain the differences by up to two orders of magnitude when
412 compared to the TEQs derived from the chemical analyses in the present study.
413 Therefore, if assuming an additive model, the instrumentally derived TEQ values based
414 only on the 16 priority PAHs clearly underestimated the real contamination of the
415 samples by AhR activating compounds. The attempts to attribute the EROD inducing
416 potency of organic sediment extracts to only 16 identified priority PAHs has often failed
417 (Khim et al., 1999; Fent and Bätscher, 2000; Hollert et al., 2002; Brack et al., 2003;
418 Qiao et al., 2006). The dioxin-like responses observed in our study were thus likely to
419 have been caused by other ubiquitous biogenic and anthropogenic PAH-like AhR
420 ligands that were not taken into account by the chemical analyses. For instance, various
421 substituted PAHs such as oxidized and sulphured PAHs have been identified as AhR
422 active chemicals in aquatic sediments by using bioassay-directed fractionation and toxic
423 identification (TIE) approaches (Brack and Schirmer, 2003). It cannot be excluded that
424 such compounds may have contributed to the AhR activities detected in our samples.
425 However, to our knowledge, the presence or not of substituted PAHs in Bizerta lagoon
426 sediments is not known.
427 As already pointed out by other authors (Jones et al., 2000, Vondracek et al., 2001,
428 Machala et al., 2001), this study further argues for the use of different times of exposure
429 in order to characterise the sample contamination by non-persistent AhR ligands and
430 stresses the need to assess IEFs at the observed duration of exposure. The screening of
431 individual PAHs confirmed that, on a concentration basis, a short exposure time led to a
432 significantly higher AhR-mediated activity (e.g. by up to two orders of magnitude for
433 B*a*A or B*b*F), thereby enhancing the sensitivity of the bioassay for the detection of
434 active PAHs at low concentrations. The decrease of AhR-mediated response with

435 increased time of exposure to PAHs has been described in other AhR expressing cell
436 lines from other species like HepG2 (Jones et al., 2000), H4IIE DR-CALUX (Machala
437 et al., 2001) or RTL-W1 cells (Bols et al., 1999). In PLHC-1 cells, the potency of
438 individual PAHs to induce EROD activity after 24 or 72 h has been previously
439 described (Fent and Bätscher, 2000; Villeneuve et al., 2002) and our results using a 24 h
440 exposure were in line with those previous studies (Fent and Bätscher, 2000). However,
441 to our knowledge, such systematic screening of the EROD induction potencies of the 16
442 priority PAHs after short-term exposure is reported here for the first time in these cells.
443 Since EROD potencies of individual PAHs were differently affected by cell
444 metabolism, the use of IEFs determined at a specific time (e.g. IEF_{4h}) should be
445 recommended for TEQ calculation of samples contaminated by weakly persistent AhR
446 agonists.
447 Lagoon sediment extracts were also screened for their ability to interfere with estrogen
448 and androgen receptor–mediated gene expression. The activities were limited since,
449 among the different sites, a significant estrogenic activity (0,22-0,29 ng E2-EQ/g dw)
450 was detected only at the MB station in the two surveys. Nonetheless, these activities
451 were of the same order of magnitude as those reported by the use of the ER-CALUX
452 assay in contaminated sediment from Zierikzee harbour (0.46 ng E2-EQ/g dw)
453 (Houtman et al, 2006) and fromm Aa station (0.22 ng E2-EQ/g dw) in Dutch rivers
454 (Houtman et al., 2007). Interestingly, the MB site was the one most contaminated by
455 PAHs and it is not unlikely that those compounds, among others, may have contributed
456 to the observed estrogenicity. Indeed, hydroxylated PAH metabolites produced by phase
457 I biotransformation, can bind to the ER and induce its transactivation in MCF-7 cells
458 (Charles et al., 2000, Fertuck et al., 2001). They may also indirectly activate the ER-
459 responsive genes through a positive cross-talk between ER and liganded AhR (Ohtake
460 et al., 2003). In MELN cells, we have recently shown that many of the PAHs studied
461 here are able to induce luciferase activity and are thus susceptible to be detected in
462 environmental mixtures using this cell line (Aït-Aïssa et al., unpublished). However,
463 both activities are not necessarily due to the same activating chemicals, as demonstrated
464 by Pillon et al (2005) who used an ER-affinity column that allowed the separation of ER
465 and AhR activities from the same sediment samples. Thus, other anthropogenic
466 chemicals that act as xeno-estrogens are likely to be present, like organo-chlorinated

467 pesticides and break down products (DDD, DDE) or PCBs and their hydroxylated
468 metabolites, which have been shown to be present in sediments at the Menzel
469 Bourguiba site (Derouiche et al., 2004; Cheikh et al., 2002). Besides xenoestrogenic
470 compounds, natural estrogenic compounds are an important source of estrogenic
471 activity in the aquatic environment (Sumpter al, 2005; Peck et al, 2004). The MB site
472 receives wastes from large urban and industrial settlements. Thus, the estrogenic
473 activities were to be expected due to both natural and synthetic hormones released from
474 municipal wastes.
475 Interestingly, anti-androgenic activity was also detected in MB extracts, at similar
476 sample concentrations as those necessary to induce estrogenic activity. Because several
477 environmental xeno-estrogens are known to possess anti-androgenic activity as well
478 (Sohoni and Sumpter, 1998), it can be expected that active endocrine disrupters, other
479 than typical AhR ligands detected in the PLHC-1 cells, are present at this station.
480 However, further studies using bioassay-directed chemical analyses will be necessary to
481 identify the nature of the bio active chemicals in the Bizerta lagoon.
482
483 In summary, the occurrence of dioxin-like, estrogenic and anti-androgenic activities by
484 using *in vitro* assays is thus reported for the first time in Bizerta lagoon sediments. A
485 first attempt to identify the nature of active chemicals showed that the 16 priority PAHs
486 significantly contributed to the measured dioxin-like activities, although a large part of
487 the EROD-inducing potencies were due to non identified PAHs or other metabolisable
488 compounds, possibly toxicologically important. The bioanalytic approach proved its
489 strength in the assessment of low concentrations of PAH incomplex environmental
490 mixtures. Furthermore, this work suggests the relevance of the implementation of
491 different exposure duration in order to differentiate between AhR-mediated activities
492 exerted by readily metabolised and persistent dioxin-like compounds. Finally, additional
493 studies using biochemical biomarkers (EROD, oxidative stress) in Gobiidea fish
494 sampled in these sites are underway in order to evaluate the environmental chemical
495 exposure of and possible impacts on benthic aquatic vertebrates in Bizerta lagoon.
496
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664

Table 1: GPS location and general characteristics of the studied sites

Sites	Chanal (CA)	Njila (NJ)	Menzel Bourguiba (MB)	Maghrawa (MR)	Menzel Jemil (MJ)	Menzel Abderrahmen (ML)	Ghar el melh lagoon (GH)
GPS coordinates	N 37 15 22 E 9 52 05	N 37 14 22 E 9 48 43	N 37 09 26 E 9 48 40	N 37 09 53 E 9 54 18	N 37 13 08 E 9 55 57	N 37 13 34 E 9 51 40	N 37 09 14 E 10 13 10
Pressures	Commercial port, ceramic industries, solid waste landfill	-	Urban, metallurgy activities, ship building, solid waste landfill	Agricultural run-off	Urban, industrial, aquaculture	Urban, solid waste landfill	-

Table 2: PAHs content (ng/g dry sediment) measured in organic extracts of sediments from the 7 studied sites sampled in summer and winter 2006[a].

Analysed PAHs	Abrev.	LOQ (ng/g)	Winter 2006							Summer 2006						
			MB	MJ	ML	CA	NJ	MR	GH	MB	MJ	ML	CA	NJ	MR	GH
Naphtalene	Nap	0.04	0.12	0.14	0.14	0.13	0.12	0.11	0.07	0.13	0.06	0.09	0.06	0.12	0.08	0.04
Acenaphthylene	Acpy	0.02	0.90	2.58	0.47	0.93	0.77	2.27	1.12	2.99	1.02	0.91	1.38	1.18	0.78	0.90
Acenaphthene	Acp	0.42	0.76	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Fluorene	Flu	0.04	0.67	0.70	0.55	0.36	0.18	0.77	nd	0.46	nd	nd	nd	0.24	0.20	0.17
Phenanthrene	Phe	0.04	22.21	1.13	8.45	5.48	nd	0.81	nd	44.05	0.14	0.44	nd	0.50	0.39	nd
Anthracene	Ant	0.10	5.03	0.13	3.37	1.40	0.34	0.20	nd	12.83	nd	0.23	0.21	nd	0.10	nd
Fluoranthene	Flt	0.06	49.04	1.71	10.72	11.10	1.04	1.26	0.82	170.33	0.99	5.84	1.55	0.36	0.53	0.21
Pyrene	Pyr	0.14	35.54	1.15	8.43	8.74	0.92	0.91	0.63	133.51	0.93	4.70	1.39	0.30	0.43	0.20
Benzo[a]anthracene	B[a]A	0.06	14.48	0.44	nd	1.68	0.43	nd	0.09	15.21	nd	0.56	0.23	0.11	nd	nd
Chrysene	Chr	0.06	7.32	0.52	nd	1.06	0.35	nd	0.08	11.23	nd	0.42	nd	0.26	nd	nd
Benzo[b]fluoranthene	B[b]F	0.50	19.10	0.79	3.27	3.05	nd	nd	nd	34.38	nd	2.40	nd	nd	nd	nd
Benzo[k]fluoranthene	B[k]F	0.50	8.10	0.77	2.06	3.13	0.74	nd	nd	8.72	nd	1.51	nd	nd	nd	nd
Benzo[a]pyrene	B[a]P	0.50	16.59	0.79	2.65	4.82	nd	nd	1.58	12.91	0.67	3.13	0.53	nd	nd	nd
Indeno[1,2,3-cd]pyrene	Ind	1.20	15.03	nd	4.68	nd	nd	nd	nd	54.86	nd	2.60	nd	nd	nd	nd
Dibenz[a,h]anthracene	DBA	1.20	1.50	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Benzo[ghi]perylene	B[ghi]P	1.20	12.66	nd	3.46	3.76	nd	nd	nd	35.92	nd	2.13	nd	nd	nd	nd
ΣPAHs (ng/g dry wt.)			209.1	10.9	48.3	45.6	4.9	6.3	4.4	537.5	3.8	25.0	5.4	3.1	2.5	1.5

a: LOQ: Limit of quantification determined as described in Materials and Methods; n.d.: below quantification limit.

Table 3: Maximal EROD activities, EC50 values and induction equivalency factors (IEF) relative to B[a]P (IEF_{BaP}) and TCDD (IEF_{TCDD}) in PLHC-1 cells exposed for 4 and 24 h to individual PAHs[a].

Chemicals	EC50 (M)		Maximal EROD (pmol/min/mg)		$IEF_{BaP\ 4h}$	$IEF_{TCDD\ 4h}$	$IEF_{TCDD\ 24h}$
	4 h	24 h	4 h	24 h			
TCDD	1.07E-10	9.87E-11	29	156	30.19	1	1
DBA	1.02E-09	1.87E-08	32	192	3.66	1.21E-01	6.11E-03
B[*k*]F	1.40E-09	2.98E-08	29	143	2.94	9.73E-02	4.23E-03
B[*a*]P	4.12E-09	2.45E-07	19	51	1	3.31E-02	5.13E-04
Ind	4.47E-09	6.99E-08	35	68	8.43E-01	2.79E-02	1.64E-03
B[*b*]F	5.94E-09	2.72E-07	29	31	6.94E-01	2.30E-02	4.63E-04
Chr	1.56E-08	3.71E-07	40	177	2.92E-01	9.68E-03	3.76E-04
B[*a*]A	1.77E-08	1.42E-06	39	55	2.58E-01	8.54E-03	9.77E-05
Flu	4.76E-07	n.i.	16	n.i.	1.44E-02	4.76E-04	n.i.
Acpy	1.23E-06	n.i.	12	n.i.	5.56E-03	1.84E-04	n.i.
Pyr	1.44E-06	5.43E-06	11	10	3.58E-03	1.18E-04	3.85E-05
Flt	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
Phe	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
B[*ghi*]P	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
Acp	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
Ant	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
Nap	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.

a: n.i.: no EROD induction detected within the 0.1 nM-10 µM concentration range

Table 4: B[a]P- and TCDD-equivalent values derived from chemical analyses (BEQchem, TEQchem) and *in vitro* PLHC-1 bioassay (BEQbiol, TEQbiol) in organic extracts of Bizerta and Ghar el Mehl lagoon sediments[a].

	BEQs, 4h exposure			TEQs, 24h exposure		
Samples	Bioassay-derived BEQs (ng/g)	PAHs analyses-derived BEQs (ng/g)	ratio (%) BEQchem / BEQbiol	Bioassay-derived TEQs (ng/g)	PAHs analyses-derived TEQs (pg/g)	ratio (%) TEQchem / TEQbiol
Winter 2006						
MB	6475	77.8	1.2	5.8	90.7	1.6
ML	1566	17.9	1.1	1.8	19.5	1.1
CA	1887	17.5	0.9	1.7	18.0	1.1
MJ	859	6.5	0.8	0.8	4.3	0.6
NJ	253	2.9	1.1	n.d.	3.4	-
MR	17	0.1	0.6	n.d.	0.03	-
GH	183	3.1	1.7	n.c.	0.9	-
Summer 2006						
MB	5938	112.7	1.9	12.8	154.26	1.2
ML	751	11.7	1.6	0.7	13.71	1.9
CA	133	2.1	1.6	n.d.	0.33	-
MJ	81	1.5	1.9	n.c.	0.37	-
NJ	89	1.1	1.2	n.d.	0.12	-
MR	17	0.6	3.7	n.d.	0.012	-
GH	n.c.	0.1	-	n.d.	0.006	-

a: BEQ = B[a]P-equivalents ; TEQ = TCDD-equivalents ; n.c.: not calculated, slight EROD induction was detected but the low magnitude of the response did not allow EC_{50} determination ; n.d.: not detected.

Figure captions

Figure 1: Map of study area and location of sampling sites in Bizerta and Ghar el Melh lagoons.

Figure 2: (a) Dose-response for luciferase activation by E2 in the MELN cell line and by DHT in the MDA-kb2 cell line; (b) dose-response for EROD induction by TCDD and BaP after 4 and 24 h in the PLHC-1 cell line. Values are means of triplicates ± S.D.

Figure 3: Estrogenic and anti-androgenic activities detected in MB site sampled in (a) summer and (b) winter 2006. Results are expressed as percentage of maximal luciferase activity induced by E2 10 nM or DHT 0.3 nM in MELN or MDA-kb2 cells, respectively. Values are means of triplicates ± S.D.

Figure 4: EROD induction in PLHC-1 cells exposed for either (A-B) 4 h or (C-D) 24 h to serial dilution of sediment extracts sampled (A-C) in summer and (B-D) in winter 2006. Results are expressed as percentage of maximal EROD activity induced by TCDD 1 nM. Values are means of triplicates ± S.D.

Figure 5: Correlation between chemical analyses- and bioassays-derived (a) BEQs and (b) TEQs determined in sediments extracts after 4 h and 24 h of exposure, respectively.

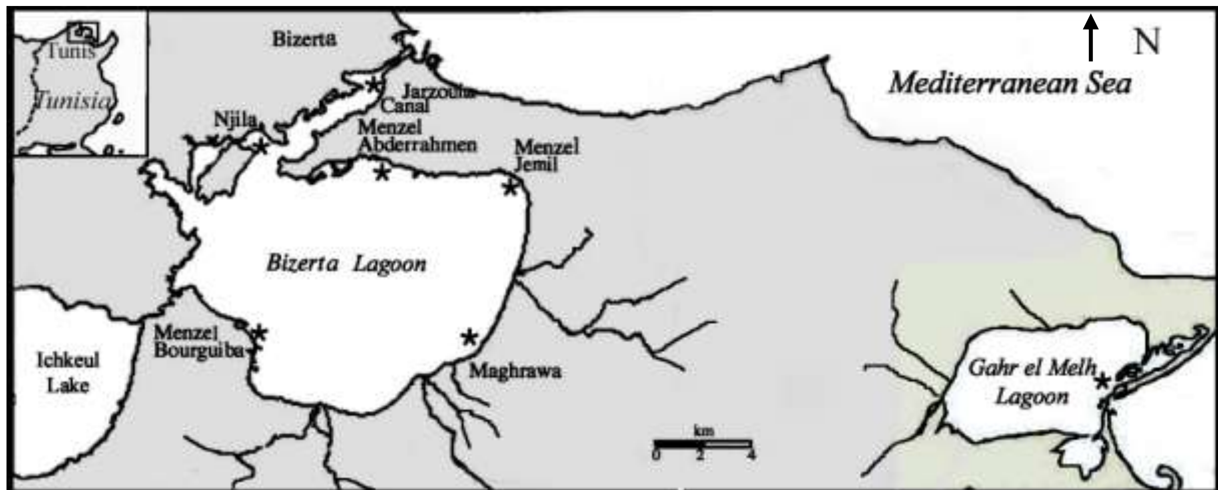
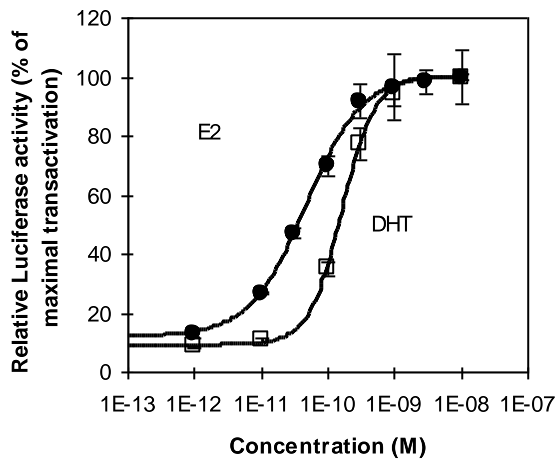


Figure 1- Map of study area and localisation of sampling sites in Bizerta and Ghar el Melh lagoons.

a- **b-**



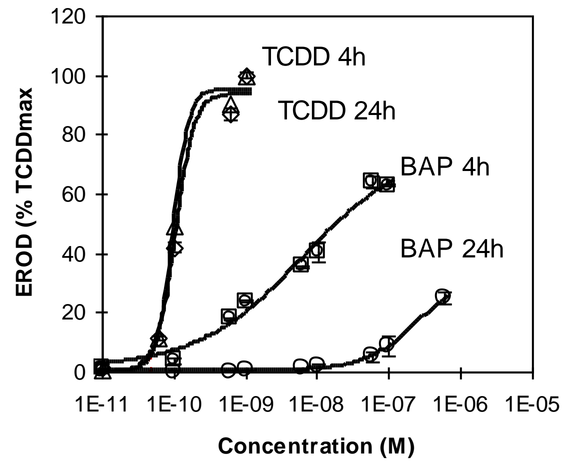
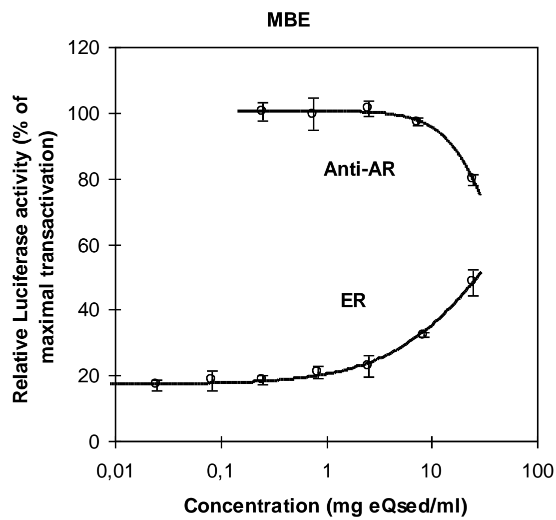


Figure 2: (a) dose-response for luciferase activation by E2 in the MELN cell line and by DHT in the MDA-kb2 cell line; (b) dose-response for EROD induction by TCDD and BaP after 4 and 24 h in the PLHC-1 cell line.

a- **b-**



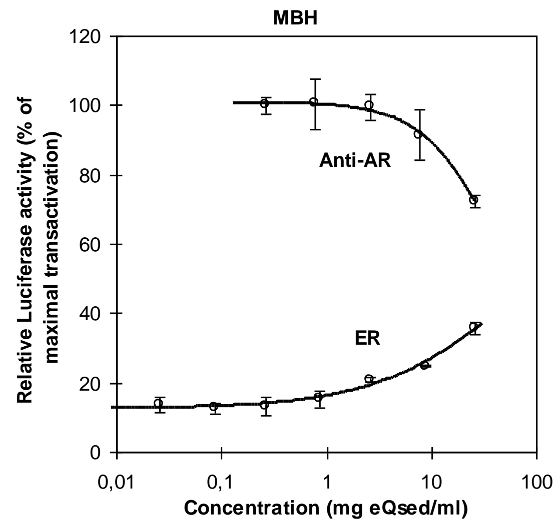


Figure 3: Estrogenic and anti-androgenic activities detected in MB site sampled in (a) summer and (b) winter 2006. Results are expressed as percentage of maximal luciferase activity induced by E2 10 nM or DHT 0.3 nM in MELN (ER responsive cells) or MDA-kb2 cells (AR responsive cells), respectively.

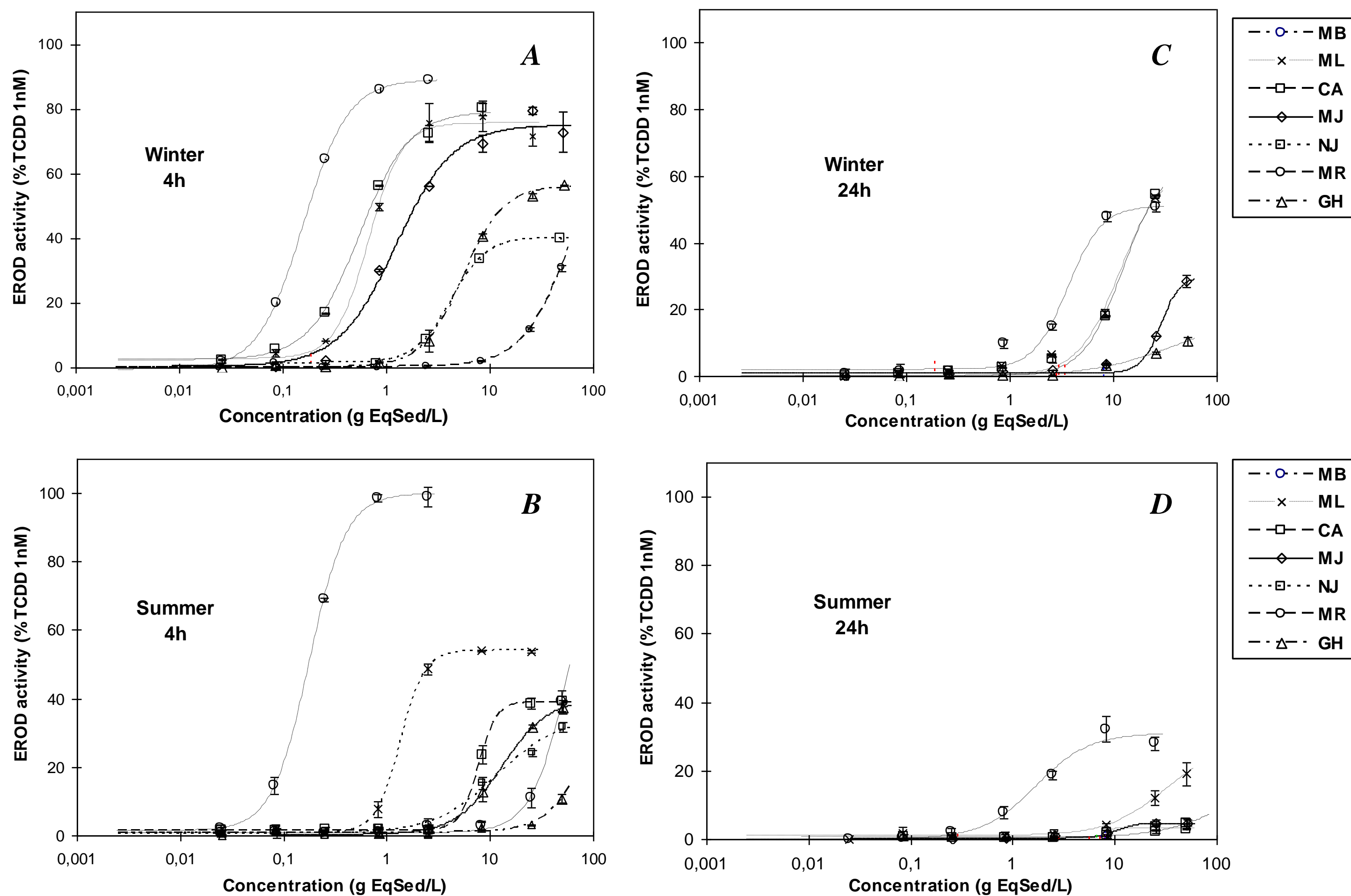
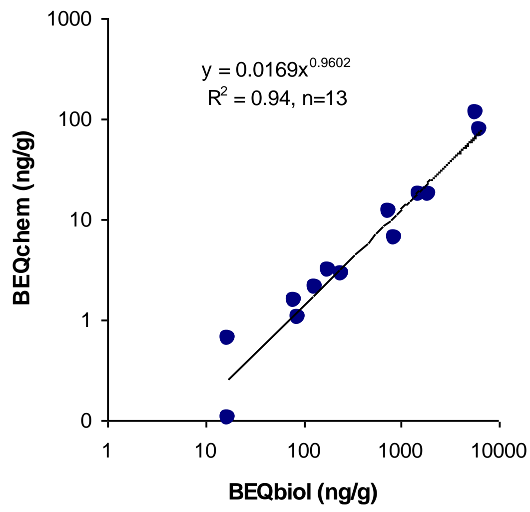


Figure 4 : EROD induction in PLHC-1 cells exposed for either (A-B) 4 h or (C-D) 24 h to serial dilution of sediment extracts sampled (A-C) in summer and (B-D) in winter 2006. Results are expressed as percentage of maximal EROD activity induced by TCDD 1 nM. Error bars (sometimes obscured by markers) indicate the standard deviation of triplicate measurements.

a-



b-

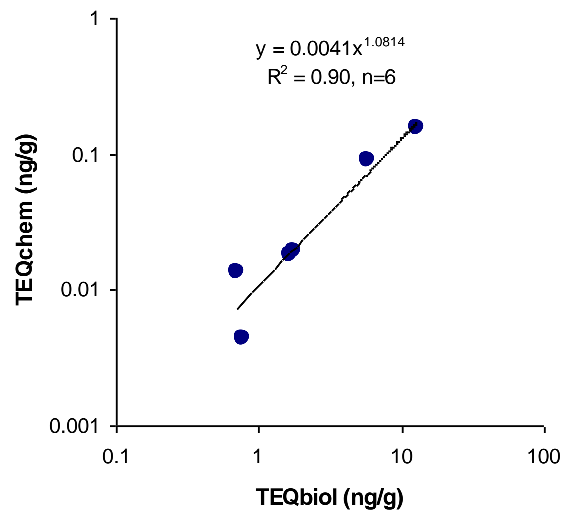


Figure 5: Correlation between chemical analyses- and bioassays-derived (a) BEQs and (b) TEQs determined in sediments extracts after 4 h and 24 h of exposure, respectively.