

Measurement of estrogenic activity in sediments from Haihe and Dagu River, China

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Abstract

Sediments from two rivers in China, the Haihe and Dagu Rivers, were examined for estrogenic activity using an estrogen receptor (ER)-mediated *in vitro* bioassay system. ER-active compounds were isolated from sediments by Soxhlet extraction, and the crude extracts were fractionated using a florisil column into three fractions. The estrogenic activity of each extract was detected by measuring luciferase activity in the human breast cancer cell line MCF-7 transfected with a luciferase receptor gene. Significant estrogenic activity was observed in each total extract. The 17 β -estradiol equivalents (E2-EQs) ranged from 8.24 to 95.28 ng E2 g⁻¹ dw. As a result, the relative estrogenic potencies of three fractions in this study descended in the order of Fraction 3 > Fraction 2 > Fraction 1. The results of the bioassay analysis indicated the heavy pollution status of these sites with estrogenic contaminants. In this study, five selected chemicals, the natural estrogens 17 β -estradiol (E2) and estrone (E1), and the xeno-estrogens 4-octylphenol (OP), 4-nonylphenol (NP), and Bisphenol A (BPA) were also analyzed using the *in vitro* bioassay. The estrogenic activity of these chemicals were E2 > E1 > NP > OP > BPA.

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1. Introduction

Some chemicals released to the environment are able to mimic the behavior of natural endogenous estrogens. And they are suspected of being responsible for an increase in the disruption of the normal physiological functions of the endocrine systems of mammals, fish, birds, reptiles and invertebrates (Golden et al., 1998; Tyler et al., 1998). These chemicals include: natural estrogens, such as 17 β -estradiol and estrone; synthetic estrogens, such as 17 α -ethinylestradiol; as well as xeno-estrogens, such as alkylphenols (including nonylphenol and octylphenol) and Bisphenol A (Gray and Metcalfe, 1997; Staples et al., 1998;

Hasselberg et al., 2004; Balch and Metcalfe, 2006). Also some persistent organochlorine pesticides (DDT derivatives, such as *o*, *p'*-DDT) have been proved to have estrogenic potency (Gaido et al., 1997; Soto et al., 1995; Bonefeld-Jorgensen et al., 2005; Greenlee et al., 2005). There are many significant classes of chemicals that have been analyzed in detail, as well as Polychlorinated Biphenyls (PCBs) (Vaccaro et al., 2005; Kitamura et al., 2005; Gierthy et al., 1997) and Polybrominated diphenyl ethers (PBDEs) (Verslycke et al., 2005), primarily due to their potential endocrine-disrupting activity. Furthermore, for a number of compounds, the endocrine disrupting potency is unknown; and in environmental matrices, many chemicals are present as complex mixtures with other compounds, which may interact to produce additive, greater than additive, or antagonistic effects (Safe et al., 1997). *In vitro* cell bioassays based on the

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mechanism of action of estrogens provide a rapid, sensitive and inexpensive solution to some of the limitations of chemical analysis. They can be useful to determine the total biological potency of a mixture of chemicals or environmental extracts as well as individual substances. The estrogenic or anti-estrogenic activity of any chemical is due to the ability of the compound to interact with the estrogen receptor (ER) of the cell. Some *in vitro* assays that act through the ER mode, such as the E-Screen-assay, the yeast estrogen screen (YES)-assay or receptor binding-assays, have been developed to screen for estrogenic or anti-estrogenic activity of chemicals (Bolger et al., 1998; Gaido et al., 1997; Payne et al., 2000). These biological methods have been previously used to detect the estrogenic activity in complex extracts from environmental samples, such as pulp and paper mill sludge and effluents, and particulate matter in air and sediments (Clemons et al., 1998; Koistinen et al., 1998). In most studies, only a few active agents were identified due to the complex composition of the samples (Koh et al., 2005; Legler et al., 2002).

The Haihe River, the largest water system in northern China, flows through the Tianjin area. The Tianjin region has a long history as an industrial city and has a population of 9.2 million. Industrial and domestic waste waters from the city and agricultural effluent discharge into the Haihe and Dagu Rivers which carries the water to the Bohai Sea at Tanggu influx (Yuan et al., 2004). The aim of this study was to determine the *in vitro* estrogenic activity in sediments collected from five locations along the Haihe and Dagu Rivers. There are no previous records of the estrogenic potential of contaminants present as complex mixtures in these areas. In this study, the estrogenic effect was assessed with a constructed ER-responsive cell line. In addition, several selected chemicals, namely 17 β -estradiol, estrone, octylphenol, nonylphenol and Bisphenol A, were tested for estrogenic potency in the *in vitro* bioassay.

2. Materials and methods

2.1. Reagents

The following standards and reagents were used in this study: 17 β -estradiol (E2, 98%) and estrone (E1, 99%) (Sigma, Germany), 4-octylphenol (OP, >98%) and 4-nonylphenol (NP, >98%) (Tokyo Kasei Kogyo Co. LTD, Japan), Bisphenol A (BPA, 97%) (Acros Organics, NJ), silica gel 60, column chromatography (Merck, Germany), anhydrous sodium sulfate (Na₂SO₄), acid-

activated copper powder, 99% (Fluka), activated florisil, 60–100 mesh size (Sigma, St. Louis, Mo, USA), *n*-hexane (99%) and methanol (Merk, Germany), and dichloromethane (DCM, 99%) (TEDIA, USA).

The following were used for cell culturing and testing: Dulbecco's Modified Eagle's Medium (Sigma), the LIVE/DEAD® Viability/Cytotoxicity Kit (L-3224) (Molecular Probes, Invitrogen, Eugene, OR), the luminescence receptor gene assay system (LucLite™ kit) (Packard BioScience, Netherlands). Milli-Q (MilliQ-185, USA) purified water was used throughout. The bioassay cell culture, previously used to measure estrogenic activity in sediment samples, was the human breast cancer cell line MCF-7 that has been stably transfected with a receptor gene to allow expression of the luciferase enzyme under the control of an estrogen-regulatory element (Khim et al., 1999).

2.2. Sampling

Sediment samples were collected from five sites along the Haihe and Dagu Rivers on November 15–16, 2003 (Fig. 1). The Jinggangqiao (Jingq) sampling site lies in an urban district and is an important crossing point of several branches of the Haihe River. Site Zhahou (Zhah) is in the estuary of the Haihe River. Dagu River is an important drainage river in Tianjin, and sampling sites Xinzhuangzi (Xinzh), Daguzhong (Dagz) and Dagukou (Dagk) are in the Dagu River.

Samples of sediments (<10 cm depth) were collected with a stainless steel grab and then placed into hexane-rinsed glass jars with aluminum foil coverings. All sediment samples were immediately transferred to the laboratory and kept at –20 °C in the refrigerator. The samples were freeze-dried (FD-1, China), homogenized using a blender with stainless steel blades, and passed through a 63- μ m sieve before extraction.

2.3. Sediments extraction and fractionation

Five g of the freeze-dried sediment was mixed with 10 g of silica gel 60, 15 g of Na₂SO₄ to remove residual moisture, and 10 g of copper powder to remove the sulfur. The sediment was then refluxed with a mixture of 300 mL DCM and hexane (3:1) for 16 h in a Soxhlet apparatus. After extraction, the solvent-soluble material was back extracted into hexane by rinsing the solvent three times with 15 mL hexane in a separating funnel. The extract was rotary evaporated (~40 °C) (Buchi, Switzerland) and concentrated to 1 mL.

The concentrated sediment extract was further fractionated into three fractions according to the method described previously (Hilscherova et al., 2000; Khim et al., 1999). Briefly, the extracts were added to a column that contained 10 g of activated florisil and 100 mL of hexane was eluted to obtain Fraction 1 that contained the PCBs and a portion of the PCDD/PCDFs. A mixture of hexane containing 20% DCM was next used to elute organochlorine (OC) compounds, PAHs, alkylphenolethoxylates and the remaining PCDD/PCDFs to obtain Fraction 2. Fraction 3 was eluted with a mixture of DCM containing 20% methanol and contained the most polar compounds. The MCF-7 bioassay was performed on each fraction to calculate the contribution that each of these fractions made to the overall ER-mediated activity. The fractions were then rotary evaporated (~40 °C) and concentrated to 1 mL.

2.4. Cell culture conditions and bioassay

Bioassay of the estrogenic activity of each sample with the MCF-7 cell line was done as previously described (Khim et al., 1999). In brief, 60 interior wells of a 96-well culture plate were each seeded with 250 μ L cell suspension at a density of about 60,000 cells well⁻¹. Cells were cultured overnight under aseptic conditions in a humidified CO₂ incubator at 37 °C and 5% CO₂ prior to dosing. Six dilutions of E2 were prepared at concentrations of 0.001, 0.003, 0.01, 0.03, 0.1 and 0.3 nM. The dilutions were then added to the wells at three wells per dilution. Extract dilutions were diluted by the same method to give six dilutions and added to wells in the same way. Solvent control was added to three wells. Cells were then incubated for 72 h in a humidified CO₂ incubator at 37 °C and 5% CO₂ after which the luciferase assay was carried out. Briefly, MCF-7 cells were washed three times with phosphate-buffered saline (PBS) buffer, after removal of culture medium, and then lysed with 75 μ L PBS buffer containing Ca²⁺ and Mg²⁺. Then 75 μ L luciferase assay reagent was added to each well. Plates were incubated for 20 min at room temperature in a dark place. Luciferase activities

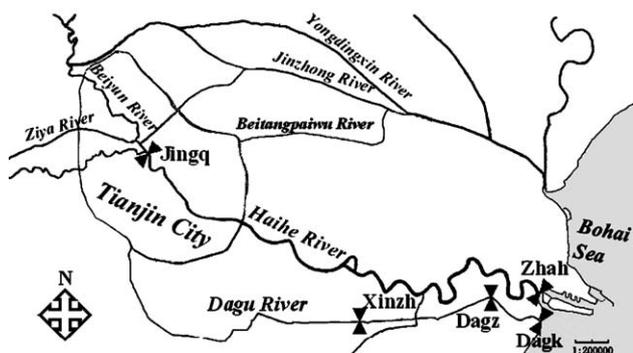


Fig. 1. Sediment sampling locations (marked with \blacktriangle) along the Haihe and Dagu Rivers, China.

were measured with an automate luminometer (Dynatech ML 3000 Luminometer; Chantilly, VA). Extract responses, expressed as mean relative luminescence units (RLUs) of the three replicate wells, were converted to relative response units, expressed as a percentage of the maximum response observed for E2 (% E2_{max}). The toxic equivalent was calculated according to the method described by Villeneuve et al. (2000).

Parallel to the MVLN assay the extract of interest was tested for cytotoxicity towards the MCF-7 cells using a LIVE/DEAD[®] Viability/Cytotoxicity Kit. Only a dose range without any obvious cytotoxic effect was applied to the bioassay (Hilscherova et al., 2002).

2.5. Chemical analysis

In this study, the concentrations of OP, NP and BPA in samples were determined by GC–MS using an Agilent 6890 GC coupled with a 5973 Mass Selective Detector. The analytical methods and GC–MS–SIM conditions were described previously (Jin et al., 2004). The levels and distribution patterns of the selected organochlorine pesticides (OCPs=*p,p'*-DDT, *o,p'*-DDT, *p,p'*-DDE, *p,p'*-DDD, α -, β -, γ - and δ -HCH) in surficial sediments were investigated by means of gas chromatography (HP-1 fused silica capillary column, 50 m length \times 0.32 mm i.d. \times 0.25 μ m film thickness) coupled with a micro-electronic capture detector (GC- μ ECD). The temperatures of injector and detector were 220 and 300 °C, respectively. Oven temperature increased from 80 °C (held for 1 min) to 180 °C (held for 2 min) at the rate of 50 °C/min, followed to 230 °C (held for 2 min) at the rate of 5 °C, and then programmed to 270 °C at 10 °C/min, held for 15 min (Yang et al., 2004, 2005).

3. Results and discussion

3.1. In vitro bioassay

The mean % values of luciferase response/E2_{max} response were used to generate the dose–response curves. The dose–response curve of E2 used as a standard in the MVLN-*luc* bioassay was sigmoidal in appearance: $y = y_0 + a/[1 + \exp(x_0 - x)/b]$. Luciferase induction was reproducible with coefficients of variation (CV) less than 20%. The limit of detection (LOD) and EC₅₀ for luciferase induction by E2 were 0.0013 ± 0.0004 and 0.0046 ± 0.0008 nM, respectively. Typical results (Total extract and fractions of Xinzhe Sample) obtained with the MVLN-*luc* analysis of sediment extracts and the fractions are given

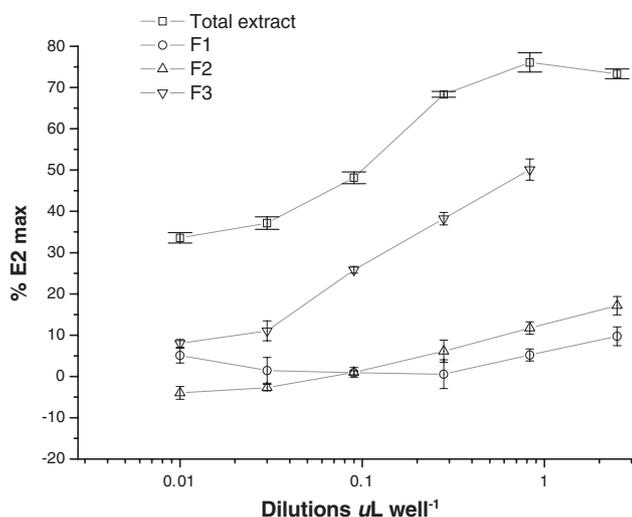
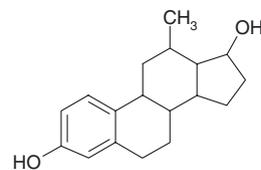
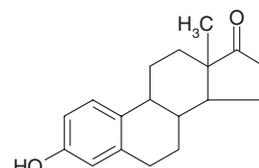


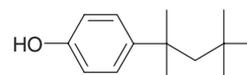
Fig. 2. Typical dose–response curves for the induction of luciferase activity in the MVLN-*luc* assay by the total sediment extracts and sediment extract fractions, F1, F2 and F3. Luciferase induction is expressed in terms of the percentage of luciferase activity relative to that of 0.3 nM E2 (E2_{max}). Values represent the mean \pm S.D.; $n=3$.



17 β -estradiol (E2)



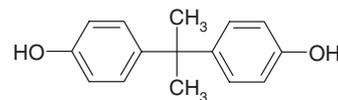
estrone (E1)



4-octylphenol (OP)



4-nonylphenol (NP)



Bisphenol A (BPA)

Fig. 3. Structural formulae of the tested natural estrogens and xeno-estrogens.

(Fig. 2). The estrogenic activity in samples derived from bioassay analysis was expressed as E2 equivalent quantity (E2-EQs, ng E2-eq g⁻¹ dw), which is based on the standard curve of E2. Significant responses were defined as those greater than three times the standard deviation of the response obtained from the solvent control.

3.2. Estrogenic activity of selected chemicals

Five chemicals with known estrogenic activity, namely the natural estrogens 17 β -E2 and E1, and the xeno-estrogens NP, OP and BPA, were tested in the MVLN-*luc* assay. Their structural formulae are shown in Fig. 3. The luciferase activity was detected at the six concentrations in the bioassay and their dose–response curves are shown in Fig. 4. The most sensitive estrogenic dose–responses were found with the natural estrogens, E2 and E1. The xeno-estrogens NP and OP produced 50%-E2_{max}-responses at a concentration of about 1×10^4 nM, and BPA was weakly estrogenic at a concentration of about 1×10^5 nM.

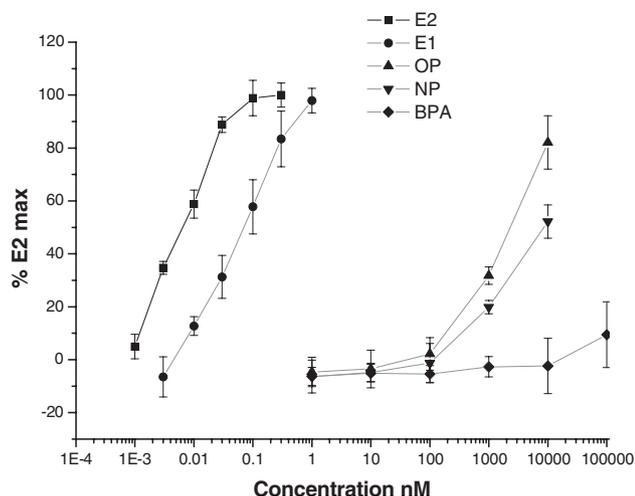


Fig. 4. Response in the in vitro bioassay toward 17β-E2, E1, NP, OP and BPA. Luciferase induction is expressed in terms of the percentage of luciferase activity relative to that of 0.3 nM E2 (% E2_{max}). Values represent the mean ± S.D.; n = 3.

These three xeno-estrogens at low concentrations showed responses lower than the response of the solvent control. Both NP and OP were cytotoxic to the MCF-7 cells at a concentration of about 1 × 10⁵ nM.

The relative potencies (E2=1) were determined as EC₅₀-values from the dose–response curve. The EC₅₀ values and the relative potencies (REP) compared to E2 of the selected chemicals are summarized in Table 1. E1 was about 15 times less potent, NP about 1 × 10⁵ times less potent and OP about 3 × 10⁵ times less potent than E2. The observed relative estrogenic potencies of E2, E1, NP and OP are in agreement with reference data (Gutendrof et al., 2001; Legler et al., 2002; Van den Belt et al., 2004). These chemicals were previously analyzed in another in vitro bioassay using a recombinant yeast cell culture. The reported relative potency of NP in the recombinant yeast culture assays was 7 × 10⁻³ (Routledge and Sumpter, 1996) and 2.5 × 10⁻⁵ (Rutishshauser et al., 2004), that of E1 was 9 × 10⁻² (Nick et al., 1997), and that of OP was 7.8 × 10⁻⁶ (Rutishshauser et al., 2004). An interesting observation is the estrogenic activity of BPA in the yeast culture assay which had a relative potency of 1.1 × 10⁻⁴ and was the most potent in Rutishshauser et al.'s (2004) study among NP, OP and BPA. In contrast, Gutendrof et al. (2001) reported a relative potency of 2.5 × 10⁻⁵ in the MVLN-cells assay for BPA, but in this study, BPA showed weak estrogenic activity at a high concentration of 1 × 10⁵ nM. As a result, the relative estrogenic potencies of selected chemicals in this study descended in the order of E2 > E1 > NP > OP > BPA.

Table 1
Comparison of EC₅₀ and the relative potencies (REP) obtained in this study and in other studies

Chemical	Values this study		Published values	
	EC ₅₀ , nM	REP	EC ₅₀ , nM	REP
E2	4.6 × 10 ⁻³	1	0.015 ^a or 0.005 ^b	1
E1	0.069	0.06	0.078 ^a or 0.48 ^b	0.2 ^a or 0.01 ^b
OP	1484	3 × 10 ⁻⁶	4286 ^c	1.4 × 10 ^{-6c}
NP	4619	1 × 10 ⁻⁶	463 ^a or 400 ^b	3 × 10 ^{-5a} or 1.25 × 10 ^{-5b}
BPA	LE	LE	200 ^b	2.5 × 10 ^{-5b}

REP of E2 was determined as 1.

LE: BPA did not reach 50% of max E2 response in bioassay.

^a Values are from Van den Belt et al. (2004).

^b Values are from Gutendrof et al. (2001).

^c Values are from Legler et al. (2002).

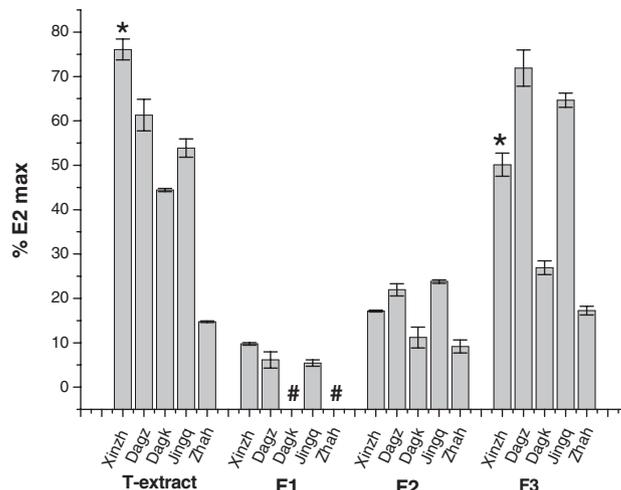


Fig. 5. Maximal luciferase induction in the MCF-7 assay elicited by total extracts and individual fractions separated by florisil column chromatography (F1: Fraction 1, F2: Fraction 2 and F3: Fraction 3). Response magnitude is expressed as percentage of E2_{max}. *: The greatest testing dilution is cytotoxic to the MCF-7 cells; #: The response is lower than the significant response level.

3.3. Estrogenic activity of sediment samples

The results of bioassay measurement of estrogenic activity using MVLN cells to the total and fractionated extracts of surface sediments from Haihe and Dagu Rivers are shown in Fig. 5. Significant induction of luciferase activity was observed with total extracts of all sediments. The maximal induction (% E2_{max}) caused by extracts was between 14.7% and 76.1% of the maximal induction elicited by E2. Three samples elicited luciferase activity that exceeded 50% E2_{max}. The highest activity was observed at location Xingzh near which are many chemical factories. Fractionation of sediment extracts showed different estrogenic activities in the bioassay. The polar fraction (F3) from sediment extract demonstrated higher estrogenic potency than mid-polar and non-polar fractions (F2 and F1). The mean response was 16.7% E2_{max} for F2 and 46.3% E2_{max} for F3. F1 showed the lowest response among the three fractions and the responses of Dagk and Zhah were lower than a statistically significant response. Cytotoxicity, determined by the LIVE/DEAD[®] Viability/Cytotoxicity Kit, was observed at the greatest testing concentration (2.5 μL well⁻¹) of total extract and F1 of the Xinzh sample. The E2 equivalent values in the total and fractionated extracts of sediment samples are shown in Table 2. Estrogenic activity was observed in all five samples collected from the Haihe and Dagu Rivers. The E2 equivalents ranged from 5.93 to

Table 2
Estrogenic equivalents (E2-EQs, ng E2 g⁻¹ dw sediments) determined by bioassays in total crude sediment extracts (T), and in extract fractions (F1, F2 and F3)

Sediment site	Estrogenic equivalents, ng E2 g ⁻¹ dw sediment			
	T	F1	F2	F3
Jingq	37	5.3	12.2	56.5
Zhah	8.2	LS	6.4	9.3
Xinzh	95.3	6.6	9.3	32.2
Dagz	49.3	5.9	11.3	77.7
Dagk	37	NS	7.1	13.7

LS: Response of sample lower than the significant response.

Table 3

Published values of the concentrations of OP, NP, BPA (ng L^{-1}) in water and concentrations of OCPs (ng g^{-1} dw) in sediments collected from sampling sites

Sediment site	OP ^a	NP ^a	BPA ^a	ΣHCH^b	ΣDDT^b
Jingq	78.4	414	126	19	80.2
Zhah	66.9	701	107	2.67	11.9
Xinzh	81.3	1056	109	34.3	83.5
Dagz	78.6	944	97	102	20.6
Dagk	71.7	485	102	141	3.6

^a Values are from Jin (Jin et al., 2004).

^b Values are from Yang (Yang et al., 2005).

95.28 ng E2 g^{-1} dw. The results were about 5 to 10 times higher than those reported from an area of the Korean coast (Koh et al., 2005) and from a Czech Republic river (Hilscherova et al., 2002).

The compounds separated to F1, including PCDDs, PCDFs, and PCBs, are known to elicit effects mediated by the aryl hydrocarbon receptor (AhR). It has been reported that some PCBs and their hydroxylated metabolites could act as weak estrogens. Our previous studies indicated that concentrations of total PCBs in the sediments were relatively high, up to about 154 ng g^{-1} dw in the Dagk site (Zhang, 2004). In this study, F1 didn't show high estrogenic potency perhaps due to the AhR-mediated anti-estrogenicity of these compounds that has been reported along with complex interactions between ER and AhR signal transduction (Navas and Seger, 1998; Safe, 1995).

Hilscherova et al. (2002) reported that the estrogenic activity was mainly present in F2. This fraction contained PAHs, their derivatives, organochlorine pesticides and alkyl phenols. PAHs were the major chemical group found in this fraction (Wang et al., 2003). Several PAHs, including benzo[α]pyrene (BaP) and benz[α]anthracene (BaA), or their oxygenated derivatives, have been shown to act as estrogenic compounds in ER-regulated receptor gene assays (Chaeles et al., 2000). Recently, several other PAHs, including fluorine, fluoranthene, pyrene, chrysene, phenanthrene and anthracene, were found to act as very weak inducers of ER-mediated activity in MCF-7 cells (Vondráček et al., 2002). Alkyl phenols, such as NP and OP, have been reported to be estrogenic in both in vitro and in vivo experiments, and pesticides, such as *o,p'*-DDT, have also been shown to elicit weak estrogenic activity. Relatively high concentrations of OP, NP, BPA and selected organochlorine pesticides were found at the sampling sites (Table 3). In a previous study (Shao et al., 2004) a high level of vitellogenin (VTG), a possible biomarker of estrogen (Sumpter and Jobling, 1995), was detected in all the male sea catfish collected from the Zhah site. Chang et al. (2003) compared the effects of BPA, NP and OP on in vitro VTG synthesis in primary cultures of hepatocytes of Chinese minnow *Phoxinus oxycephalus*. The results indicated that exposure to 10^{-5} M BPA and 10^{-4} M NP and OP induced significant in vitro VTG synthesis. But exposure to high concentrations of these three compounds in vivo were required to induce VTG synthesis. Van den Belt et al. (2003) found significant VTG induction in rainbow trout exposed to NP, OP and BPA at concentrations of 100, 30 and 1000 $\mu\text{g L}^{-1}$, respectively. Other studies have reported that 1 $\mu\text{g L}^{-1}$ NP (Schwaiger et al., 2002) and 500 $\mu\text{g L}^{-1}$ BPA (Lindholm et al., 2000) could induce significant levels of VTG in male rainbow trout. The concentrations of NP, OP and BPA in the water from the Zhah site were much lower than the concentrations that could induce VTG. Our study indicated there has been heavy pollution of this site with other estrogenic contaminants. We also found that concentrations of BPA in sediments of Xinzh and Dagz were 2 to 3 times higher than those in water. Aquatic sediments serve as a kind of enrichment medium for a number of contaminants and thus can act as an integrative measure of exposure of the aquatic ecosystem to these

contaminants (Berggren et al., 1999; Hurst et al., 2004). In our study, the greatest E2 equivalent values were observed in F3 and the results suggest the presence of estrogenic compounds in F3. The estrogenic potency of a number of substances in this group of chemicals in F3 is unknown. For the sites Jingq, Dagz and Zhah, the E2 equivalent values of F3 were higher than the values of the total extracts. Assuming that the contents of the fractions were exactly equal to their content in the total extracts then the results indicate that while F3 contained estrogenic activity, depending on the site, F1 and F2 contained substances that were either antagonistic, synergistic or of no effect on that activity. These results no doubt indicate that some chemicals in the mixture produce estrogenic effects and others can boost or be antagonistic to estrogenic activity, or have no effect.

Some toxic compounds, such as organochlorine pesticides (OCPs), xeno-estrogens (including 4-*tert*-octylphenol, 4-nonylphenol and bisphenol A), PCBs, PCDDs/PCDFs and mercury, have been reported in the previously studies in this area (Jin et al., 2004; Shi et al., 2005; Wan et al., 2005; Yang et al., 2005; Zhang, 2004). However, few data are available to indicate the level of contamination of estrogenic compounds in these rivers. In vitro MCF-7 bioassay has been shown to be a useful tool for assessing the estrogenic activity of complex environmental mixtures. The estrogenic activities in sediments from the Haihe and Dagu Rivers were significant. However, the application of the bioassay cannot quantify specific compounds. To provide an estimate of the relative level of contamination, it is reasonable to use bioassays combined with instrumental analytical approaches.

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