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ASSESSMENT OF HORMONAL ACTIVITIES AND GENOTOXICITY OF INDUSTRIAL EFFLUENTS USING IN VITRO BIOASSAYS COMBINED WITH CHEMICAL ANALYSIS

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Abstract—Wastewaters from various industries are a main source of the contaminants in aquatic environments. The authors evaluated the hormonal activities (estrogenic/anti-estrogenic activities, androgenic/anti-androgenic activities) and genotoxicity of various effluents from textile and dyeing plants, electronic and electroplate factories, pulp and paper mills, fine chemical factories, and municipal wastewater treatment plants in the Pearl River Delta region by using in vitro bioassays (yeast estrogen screen [YES]; yeast androgen screen [YAS]; and genotoxicity assay [umu/SOS]) combined with chemical analysis. The results demonstrated the presence of estrogenic, anti-estrogenic, and anti-androgenic activity in most industrial effluents, whereas no androgenic activities were detected in all of the effluents. The measured estrogenic activities expressed as estradiol equivalent concentrations (EEQs) ranged from below detection (3 of 26 samples) to 40.7 ng/L, with a mean of 7.33 ng/L in all effluents. A good linear relationship was found between the EEQs measured by YES bioassay and the EEQs calculated from chemical concentrations. These detected estrogenic compounds, such as 4-nonylphenol and estrone, were responsible for the estrogenic activities in the effluents. The genotoxic effects expressed as benzo[a]pyrene equivalent concentrations (BaP EQs) varied between below detection and 88.2 μg/L, with a mean of 8.76 μg/L in all effluents. The target polycyclic aromatic hydrocarbons were minor contributors to the genotoxicity in the effluents, and some nontarget compounds in the effluents were responsible for the measured genotoxicity. In terms of estrogenic activities and genotoxicity, discharge of these effluents could pose high risks to aquatic organisms in the receiving environments. Environ. Toxicol. Chem. 2012;31:1273–1282. © 2012 SETAC

Keywords—Hormonal effects Environmental estrogens Genotoxicity Industrial effluents The Pearl River Delta region

INTRODUCTION

In recent years, water pollution has become a serious problem for public health and aquatic ecosystems in some countries because of discharge of industrial and domestic wastewaters into aquatic environments. Industrial effluents, as undesirable byproducts of economic development and technological advancement, are considered an important source of various pollutants to the aquatic ecosystem [1–3]. With the use of many synthetic chemicals in industrial sectors, they may reach the environment with the effluents, which can lead to adverse effects on aquatic organisms and humans [4]. Therefore, understanding the potential hazards in industrial effluents is essential.

Among the reported pollutants in wastewaters, endocrine-disrupting compounds (EDCs) and genotoxic compounds are of major conern. Some EDCs, such as 4-nonylphenols (4-NP) and bisphenol-A (BPA), have been detected in industrial and municipal wastewaters [5–7], which can disturb the normal functioning of hormonal systems in wildlife and human beings [8]. In addition, some genotoxic compounds, such as polycyclic aromatic hydrocarbons (PAHs), have been found in industrial effluents, which may induce DNA damage or mutations and increase the risk of getting cancer for animals and humans [9–11].

Both chemical analysis and bioassays can be used to assess and quantify the toxicity of industrial effluents [12,13]. However, by chemical analysis approach alone, only limited chemical concentration information can be obtained from the analyzed wastewaters, with little understanding of potential adverse effects on the environment [12]. Fortunately, bioassays can integrate the effects of all pollutants and show the integrated activity of a whole effluent [14–16]. Therefore, in vitro bioassays combined with chemical analysis could provide better understanding of the risks of chemicals and potential impacts of industrial wastewaters on organisms in the environment [17,18].

A variety of bioassays, including the genotoxicity assay (umu/SOS test), Ames test, comet or single cell gel electrophoresis assay, Microtox bioluminescence test, and micronucleus as well as the anaphase aberration assays, have been used to detect the mutagenic activity of industrial effluents and surface waters [19–23]. Among these bioassays, the umu/SOS assay, a sensitive in vitro method for genotoxicity screening, has been increasingly applied to rapid screening of mutagens and carcinogens in environmental samples [24–27]. As for endocrine-disrupting effects, some in vitro bioassays, such as estrogen screen (E-SCREEN), yeast estrogen screen (YES), and estrogen-responsive chemically activated luciferase, have been developed to evaluate integrated estrogenic activity of environmental samples [28–30]. At the same time, in vitro bioassays such as androgen screen (A-SCREEN) [31,32], yeast androgen screen (YAS) [33], and androgen receptor (AR) binding assays [34,35], have also been developed to assess the androgenic activity of environmental samples [36].

The Pearl River Delta region is one of the fastest economic growth regions in China, with typical manufacturing industries such as electronic, textile, paper making, and fine chemical industries. Approximately 417 million tons of effluents are generated annually from these industries, with most of the

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wastewaters being discharged into the Pearl River system. The Pearl River is an important drinking water source for cities and towns, including Guangzhou, Hong Kong, and Macau, in the Pearl River Delta region. Thus, assessing the hazards of these industrial effluents and identifying the toxicants, especially those chemicals that may have endocrine-disrupting effects and mutagenic and carcinogenic effects, is essential.

The objective of this study is to investigate hormonal activities and genotoxicity of various industrial effluents by using an in vitro bioassay in combination with chemical analysis. Seven selected estrogenic compounds, including 4-t-octylphenol (4-t-OP), 4-NP, BPA, estrone (E1), estradiol (E2), diethylstilbestrol, and ethinyl estradiol, and 16 U.S. Environmental Protection Agency (U.S. EPA) PAHs in the effluents were determined using gas chromatography-mass spectrometry (GC-MS), whereas unknown pollutants in the effluents were scanned by GC-MS. The estrogenic and anti-estrogenic activities of industrial effluents were measured using the YES bioassay and yeast anti-estrogen screen (YAES) bioassay, respectively, and the androgenic and anti-androgenic effects were determined using the YAS bioassay and yeast anti-androgen screen (YAAS) bioassay, respectively. The genotoxicity of the industrial effluent was determined by umu/SOS with and without the S9 active system.

MATERIALS AND METHODS

Sample collection

Effluent samples were collected in the Pearl River Delta region from 26 wastewater treatment plants of five industries, including municipal wastewater treatment plants (Fig. 1). Of the 26 industrial effluent samples collected, samples TD-1, TD-2, TD-3, TD-4, and TD-5 were from five textile and dyeing plants, samples P-1, P-2, P-3, P-4, P-5, P-6, P-7, and P-8 were from eight pulp and paper mills, samples MW-1, MW-2, MW-3, and MW-4 were from four municipal wastewater treatment plants, samples E-1, E-2, E-3, E-4, E-5, and E-6 were from six electronic and electroplate factories, and samples FC-1, FC-2, and FC-3 were from three fine chemical factories.

These effluents were collected in 1-L amber glass bottles, which were all prerinsed with effluents three times before sample collection. Approximately 50 ml methanol and 400 ml 4 M H_2SO_4 were added to each 1-L bottle to adjust the pH to 3.0. The collected samples were transported in coolers to the laboratory and stored at 4°C for a maximum of 48 h before solid-phase extraction. Eight replicates of the effluent samples were collected from each industrial effluent discharge outlet. Four replicates were used for the bioassay without any internal standards (two for YES and YAS bioassays, and two for umu/SOS assay), and another four replicates were spiked with internal standards for chemical analysis (two for EDC assay, and two for PAH analysis).

Solid-phase extraction

The effluents were filtered through prebaked glass fiber filters (GF/F, Whatman 0.7 mm effective pore size, UK) before extraction. After that, the effluents for YES, YAS assay, and EDC analysis were extracted by HLB cartridges (Waters Oasis 6 ml, 500 mg) according to the method reported by Zhao et al. [37]. The effluents for umu/SOS assay and PAH analysis were extracted by the methods for EDCs by Zhao et al. [37] with a few modifications. In brief, when we eluted the solid-phase extraction cartridges, 5 ml ethyl acetate was used in addition to 7 ml methanol. The mixed eluates were blown down carefully under a gentle nitrogen stream. When the eluates were nearly dry, the eluates for chemical analysis were diluted to 1 ml dichloromethane, and the eluates for the umu/SOS assay were dissolved in 0.4 ml by dimethyl sulfoxide.

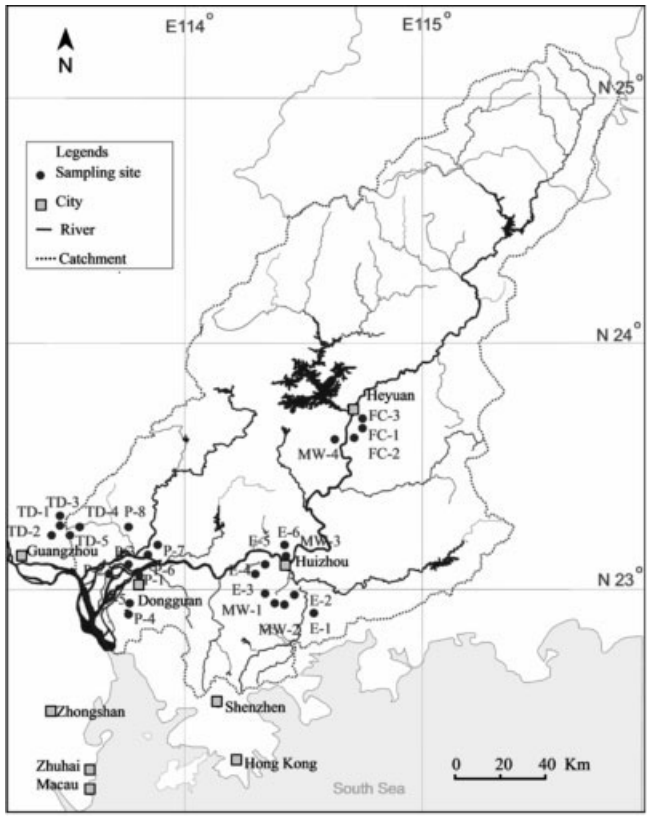

Fig. 1. Location map of sampling sites in the Pearl River Delta region of China.

Yeast estrogen screen assay

The recombinant yeast for the YES bioassay was donated by J. P. Sumpter (Brunel University, Uxbridge, UK). The YES assay was conducted according to the method developed by Routledge and Sumpter [29] with some modification, which was described in detail by Zhao et al. [38]. In each test plate, a positive control (E2 with an initial concentration of 0.2 mM exposure) and blank control (methanol exposure) were included. The estrogenic activity of a sample measured by the YES assay was expressed as an estradiol equivalent concentration (EEQ).

Yeast anti-estrogen screen assay

The anti-estrogenic activity of the effluent samples was measured by evaluating the reduction of the activity of β-galactosidase induced by the E2 standard. The methods for YAES originated from the YES assay with some modifications, which were described by Zhao et al. [39]. During the procedure, dilutions of extracted effluent samples each were treated with 10 μl E2 standard (1.36 μg/L), which is sufficient to cause submaximal estrogenic activity. A dose-dependent decrease in E2 activity signaled the presence of anti-estrogens in the wastewater. Twofold dilutions series of tamoxifen with a concentration from a maximum 742 mg/L to a minimum 0 mg/L treated with 1.36 μg/L of E2 standard were used as the positive control. All procedures for anti-estrogenic activity assay were exactly the same as the method for the YES assay previously described. The anti-estrogenic activity of a sample measured by the YAES

assay was expressed as a tamoxifen equivalent concentration (EQ).

Yeast androgen screen assay

The recombinant yeast for the YAS bioassay was also provided by Professor Sumpter. All media and strains for assays were prepared according to the original protocol for the YAS [40], and the assay procedure was modified from the procedure for the YES, which was described by Zhao et al. [39]. In the YAS assay, dihydrotestosterone (DHT) standard reagent (final concentration: 7×10^{-8} M to 5×10^{-10} M) was used as the positive control and the methanol as the blank control. The androgenic activity of a sample measured by the YAS assay was expressed as a DHT EQ.

Yeast anti-androgen screen assay

For screening of anti-androgenic activity, the method originated from the YAS assay with some modifications was described by Zhao et al. [39]. Briefly, all dilutions of extracted effluent samples were each treated with 10 μl DHT as standard reagents (43.6 μg/L; final concentration 2.18 μg/L), which is sufficient to cause submaximal androgenic activities. A serial dilution of flutamide (final concentration 27.6 mg/L to 0 mg/L) treated with DHT (final concentration 2.18 μg/L) was applied for the positive control and the flutamide standard curve. The procedure for the screening of anti-androgenic activities was exactly the same as that for YAS. The anti-androgenic activity of a sample measured by YAAS assay was expressed as a flutamide EQ.

The umu/SOS assay

The industrial effluent extracts were twofold diluted in eight series on a row of a 96-well microplate (BD Falcon), using methanol, then 4 μl of each concentration was transferred to the corresponding well on another 96-well test plate. Each microplate included negative control (dimethylsulfoxide exposure) and positive control, with benzo(a)pyrene (BaP) as indirect mutagen or 4-nitroquinoline-N-oxide (4-NQO) as direct mutagen.

The test strain *Salmonella typhimurium* TA1535/PSK1002 was provided by Yoshimitsu Oda (Osaka Prefectural Institute of Public Health). The umu/SOS assays were carried out according to the procedure described previously by Oda et al. [41], with some modifications.

An overnight culture of bacterial strain was diluted 50-fold into TGA medium (1% bactotryptone, 0.5% NaCl, and 0.2% glucose; supplemented with 50 mg/L ampicillin) and incubated at 37°C until the bacterial density reached 0.20 in OD600. Because most aromatic amines require metabolic activation to be genotoxic, an activation system was prepared containing a liver 9,000 *g* supernatant fraction (S9) from rats treated with phenobarbital and 5,6-benzoflavone, and cofactors (0.4 M $MgCl_2$, 1.65 M KCl, 1.0 M glucose 6-phosphate, 0.1 M β-nicotinamide adenine dinucleotide 2'- phosphate reduced tetrasodium salt, 0.1 M β-nicotinamide adenine dinucleotide reduced disodium salt, and 0.2 M Na-phosphate buffer [pH 7.4]) obtained from the Research Institute for Liver Disease (Shanghai).

In the bioassays performed in the absence of S9 activation, 196 μl test culture was transferred into each well of the 96-well microplate, whereas in the bioassays requiring S9 metabolic activation, 85 ml bacterial culture and 15 ml S9 mix solution were mixed with a vortex mixer, and 196 μl of the resulting mixture was added to the microplate wells. The microplates were covered with lids and incubated at 37°C with vigorous orbital shaking on a temperature-controlled shaker for 2 h, after which the cell density of the culture was measured at 600 nm wavelength (BMG Lab technologies). A total of 100 μl test culture was transferred to a new 96-well microplate. After adding 100 μl Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol, pH 7.0), 5 μl sodium dodecyl sulfate (0.01%), and 5 μl chloroform, the microplate was preincubated for 10 min at 37°C with vigorous orbital shaking (1,000 rpm). Thirty micrograms o-nitro-phenylgalactopyranoside solution (6 mg/ml, dissolved in buffer containing 0.1 M NaH_2PO_3 and 0.1 M KH_2PO_4) was added into each well to start the enzyme reaction. The microplate was incubated at 30°C on a temperature-controlled shaker for approximately 45 to 60 min and terminated by the addition of 50 μl Na_2CO_3 (1 M). After centrifugation at 12,000 *g* for 15 min, 200 μl supernatant was transferred into a new 96-well microplate. The absorbance at 420 nm and 550 nm was measured on a BMG microplate reader (BMG Lab technologies). The β-galactosidase activity was calculated according to the following equation:

$$\mathrm{IU} = \frac{1000 \times (\mathrm{OD}_{420} - 1.75 \times \mathrm{OD}_{550})}{t \times v \times \mathrm{OD}_{600}} \quad (1)$$

where IU is β-galactosidase activity, v is the dilute times for test solutions, and in this test system, v is 2.9, and t is the time of reaction (min).

The induction ratio was used to express the response value for each exposure concentration or volume. The induction ratio was calculated according to the following equation:

$$\mathrm{IR} = \frac{\mathrm{IU_i}}{\mathrm{IU_b}} \quad (2)$$

where IR is induction ratio, IU_i is the β-galactosidase activity of each concentration, and IU_b is the β-galactosidase activity for the blank control (dimethylsulfoxide exposure).

In the umu/SOS assay without metabolic activation (–S9), the direct genotoxicity of an industrial effluent sample was expressed as 4-NQO EQ, whereas with metabolic activation (+S9), the indirect genotoxicty was expressed as BaP EQ.

Chemical analysis

The concentrations of seven selected estrogenic compounds (EDCs) were determined in accordance with the method reported by Zhao et al. [37,38] using GC-MS under negative chemical ionization mode (GC-NCI-MS: Agilent 6890N gas chromatograph connected to an Agilent 5975B mass spectrometer with a chemical ionization source). The concentrations of 16 U.S. EPA PAHs in the effluents were determined using the method recommended by Barco-Bonilla et al. [42]. Some unknown chemicals in the effluent extracts were also scanned and identified with GC-MS with electron-impact ionization.

Data analysis

Bioanalytical results can be reported in terms of EQs, using a corresponding reference compound representing a group of targeted chemicals in a given assay [43,44]. The EQ represents the concentration of the reference compound required to produce the same effect as the mixture of different compounds in the sample. The dose–response curve of the reference for the control test of YES, YAES, YAS, YAAS, and umu/SOS assays can be fitted using a sigmoid equation (Hill equation) with

origin 7.5. The equation is:

$$Y = Y_{max} - \frac{Y_{max} - Y_{min}}{[1 + (C/EC50)^p]} \quad (3)$$

where Y_{max} is the maximum value that the curve infinitely closes to, Y_{min} is the minimum value that the curve closes to, p is Hill slope, EC50 is 50% effective concentration, and C is the concentration of the sample.

Equivalent concentrations were calculated by the Hill equation of the curves of the reference compound: Put response value (Y) into the Hill equation, work out C, divide by the final relative enrichment factor, and obtain an original concentration for response value. In the present study, the relative enrichment factor was calculated by the formula defined by Macova et al. [44].

Noticeably, when the EQ in the umu/SOS assay is calculated, if the induction ratio (Y) is lower than 1.5, the threshold of the genotoxic effect defined by the International Standard Organisation (ISO) guideline [45], the result cannot be accepted.

The calculated activity of a group of chemicals based on chemical analysis was expressed as the sum of a group of the same effect chemicals by multiplying the relative potency of a compound and its corresponding environmental concentration based on the addition model [38,46,47].

Quality assurance and quality control

All data generated from both the bioassay and chemical analysis were subject to strict quality control procedures. As for chemical analysis, with each set of samples analyzed, a solvent blank, a standard, and a procedure blank were run in sequence to check for background contamination, peak identification, and quantification. In addition, surrogate standards were added to all of the samples to monitor matrix effects. As for bioassay, with each set of samples analyzed, blank control and reference reagent have to be included to test the stability of the test species and the experimental environment.

RESULTS

Multiple hormonal activities in effluents

As shown in Table 1, estrogenic, anti-estrogenic, and anti-androgenic effects were observed in the effluents from five types of industries in the Pearl River Delta region, but androgenic effects were not detected in all effluents from the region. The estrogenic activities expressed as EEQs in the effluents ranged from not detected (in 3 of 26 samples) to 53.7 ng/L, with a mean of 7.33 ng/L. Big variations were observed among the same type of industrial effluents. For example, the EEQs in the effluents from textile and dyeing factories were found to range from 3.39 to 53.1 ng/L. Among the six effluent samples collected from electronic and electroplate factories (E-1 to E-6), high estrogenic activities were demonstrated in the samples E-2 and E-3, with their EEQs of 3.61 and 9.97 ng/L, respectively, whereas the EEQs of the other four effluents were all lower than 1.5 ng/L. In effluents from paper and pulp mills (P-1 to P-8), except for P-1, the estrogenic activity levels were all much lower than 1.5 ng/L, which is the predicated no effect concentration for E2. [38]

Table 1. The equivalent concentrations obtained from in vitro bioassay of industrial effluents

		μg/L					
		YES	YAES	YAS	YAAS	Umu/SOS	
Effluent	Samples	Estradiol EQ ($\times 10^{-3}$)	Tamoxifen EQ	DHT EQ	Flutamide EQ	BaP EQ (+S9)	4-NQO EQ (-S9)
TD	TD-1	16.4 ± 7.5[a]	135 ± 118	ND	1420 ± 253	5.16 ± 0.67	8.28 ± 0.64
	TD-2	11.5 ± 3.9	37.6 ± 9.5	ND	895 ± 147	4.50 ± 0.65	6.14 ± 0.90
	TD-3	53.1 ± 8.0	81.8 ± 9.8	ND	1920 ± 364	4.88 ± 0.09	23.6 ± 3.93
	TD-4	34.4 ± 12.0	90 ± 39.1	ND	1180 ± 235	7.99 ± 2.40	24.8 ± 4.40
	TD-5	3.39 ± 2.3	14.2 ± 6.9	ND	484 ± 123	ND	ND
P	P-1	6.31 ± 1.1	104 ± 99.3	ND	2150 ± 175	4.33 ± 0.17	3.7 ± 1.78
	P-2	0.45 ± 0.1	54.2 ± 21.6	ND	584 ± 80.4	4.2 ± 0.06	4.55 ± 0.45
	P-3	0.55 ± 0.2	114 ± 20.4	ND	2190 ± 178	4.09 ± 0.33	8.15 ± 0.20
	P-4	0.18 ± 0.0	42.2 ± 6.4	ND	1810 ± 422	ND	ND
	P-5	0.32 ± 0.1	18.7 ± 0.4	ND	452 ± 116	ND	ND
	P-6	0.28 ± 0.1	213 ± 21.5	ND	1860 ± 379	3.96 ± 0.49	4.93 ± 0.91
	P-7	0.11 ± 0.0	78.2 ± 20.7	ND	1690 ± 639	7.43 ± 1.71	6.45 ± 0.26
	P-8	ND	43.8 ± 1.7	ND	502 ± 80.8	4.44 ± 0.21	1.99 ± 0.56
E	E-1	ND	ND	ND	77.0 ± 22.8	3.18 ± 0.20	ND
	E-2	3.61 ± 0.7	24.8 ± 3.7	ND	446 ± 53.5	8.45 ± 0.83	19.5 ± 7.64
	E-3	9.97 ± 2.1	70.6 ± 50.6	ND	393 ± 138	8.39 ± 1.84	31.8 ± 7.40
	E-4	0.74 ± 0.4	226 ± 20.7	ND	808 ± 133	23.8 ± 2.62	43.6 ± 17.3
	E-5	0.79 ± 0.1	125 ± 8.0	ND	1740 ± 208	88.2 ± 35.1	352 ± 38.1
	E-6	0.52 ± 0.3	107 ± 32.7	ND	703 ± 236	4.63 ± 0.24	4.32 ± 1.22
MW	MW-1	3.26 ± 0.3	ND	ND	337 ± 13.7	5.18 ± 0.34	2.55 ± 0.91
	MW-2	1.28 ± 0.0	ND	ND	367 ± 116	3.92 ± 0.30	ND
	MW-3	1.57 ± 0.5	18.8 ± 11.0	ND	277 ± 49.7	4.03 ± 0.43	2.04 ± 0.61
	MW-4	ND	24.2 ± 3.0	ND	195 ± 51.2	3.80 ± 0.89	1.80 ± 1.21
FC	FC-1	0.07 ± 0.0	ND	ND	136 ± 44.9	5.18 ± 0.05	2.21 ± 0.23
	FC-2	1.18 ± 0.8	133 ± 12.0	ND	482 ± 159	10.7 ± 1.09	22.8 ± 10.8
	FC-3	40.7 ± 9.2	ND	ND	1000 ± 128	7.42 ± 0.26	58.4 ± 6.48

[a] Mean ± standard deviation ($n = 2$).

TD = textile and dyeing plants; P = pulp and paper mills; E = electronic and electroplate factories; MW = municipal wastewater treatment plants; FC = fine chemical factories; YES = yeast estrogen screen assay; YAES = yeast anti-estrogen screen assay; YAS = yeast androgen screen assay; YAAS = yeast anti-androgen screen assay; EQ = equivalent concentration (estradiol EQ in the table is abbreviated as EEQ in the text); ND = not detected.

As for the anti-estrogenic effects of industrial effluents, Table 1 shows that anti-estrogenic activities expressed as tamoxifen EQs ranged from not detected (5/26 samples) to 226 μg/L. A similar phenomenon of large variation among the same type of industrial effluents was observed for anti-estrogenic activities. For example, the tamoxifen EQs in the effluents from textile and dyeing plants varied between 14 and 135 μg/L, with a mean of 72 μg/L, whereas in the effluents from pulp and paper mills they varied between 19 and 213 μg/L, with a mean of 83 μg/L. Similarly, high anti-estrogenic activities were also found in all effluents from electronic and electroplate factories except for E-1, with their tamoxifen EQs ranging from 25 to 226 μg/L.

Although androgenic effects were not detected in all industrial effluents, anti-androgenic effects were found in all effluent samples from the Pearl River Delta region, with flutamide EQs ranging from 77 to 2,190 μg/L (Table 1). The anti-androgenic activities of the effluents were much higher than their corresponding estrogenic activities and anti-estrogenic activities. The flutamide EQs in the effluents from the paper and pulp mills ranged from 452 to 2,190 μg/L, with a mean of 14.06 μg/L. The mean flutamide EQs for the other types of effluents were as follows: 1,178 μg/L in the effluents from textile and dyeing plants, 695 μg/L in the effluents from electronic and electroplate factories, 539 μg/L in the effluents from fine chemical factories, and 294 μg/L in the effluents from municipal wastewater treatment plants.

Genotoxicity in effluents

As seen in Table 1, positive responses for mutagenic effects were found in most effluents at relative enrichment factors of 50 and 25. In the absence of metabolic activation (–S9), negative responses were found in five samples of the 26 effluent samples from the Pearl River Delta region, whereas in the presence of metabolic activation (+S9), negative responses were found in only three of the 26 samples. In four of the five effluents from textile and dyeing plants, positive responses were detected, with the 4-NQO EQs ranging from 4.50 to 7.99 μg/L for direct mutagenic effects and with the BaP EQs ranging from 6.14 to 24.8 μg/L for indirect mutagen effects. In the two effluent samples from pulp and paper mills, no positive responses for mutagenic effects were observed either with or without metabolic activation. However, the presence of metabolic activation (+S9) led to the detection of mutagenic effects in one effluent sample (E-1) from electronic and electroplate factories and another effluent sample (MW-2) from municipal wastewater treatment plants. Positive responses for mutagenic effects were noticed in all three effluent samples from the fine chemical factories, with the 4-NQO EQs ranging from 2.21 to 58.4 μg/L and with the BaP EQs ranging from 5.18 to 7.42 μg/L.

Estrogenic compounds in effluents

Five target estrogenic compounds (4-NP, 4-t-OP, BPA, E1, and E2) were detected in the effluents from the industrial sectors in the Pearl River Delta region (Fig. 2). The other two compounds (diethylstilbestrol and ethinyl estradiol) were found below the method limits of quantification in the effluents. Both E1 and E2 were only found in 14 and six of the 26 effluent samples, respectively. These two natural estrogens detected in the industrial effluents originated from the workers working in the industrial parks. For the three industrial chemicals (4-t-OP, 4-NP, and BPA), much higher concentrations were found in industrial effluents than in municipal wastewater effluents, with a few exceptions from the electronic industry (Table 2). As for

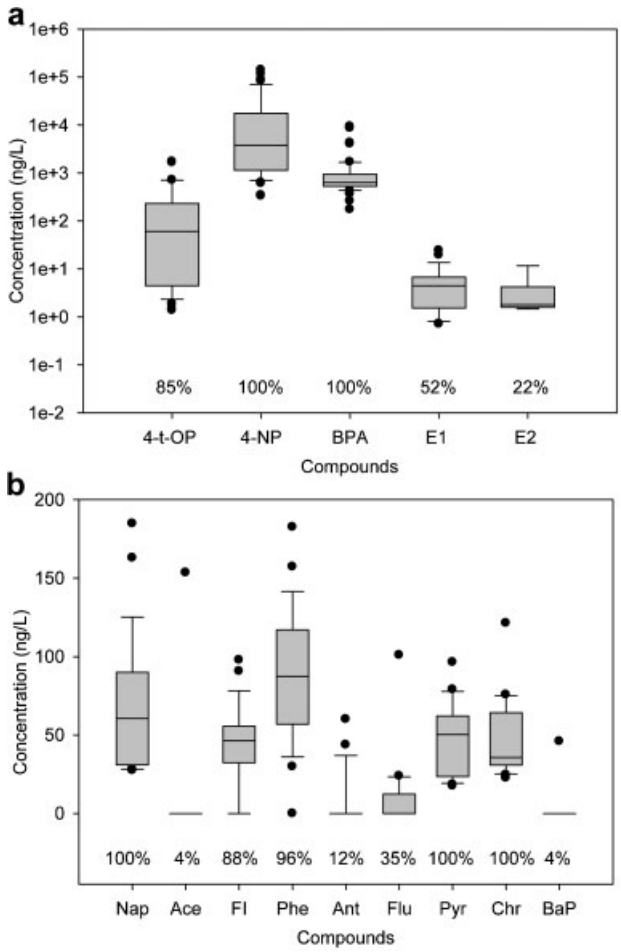

Fig. 2. Box plots for the estrogenic compounds (**a**) and polycyclic aromatic hydrocarbons (PAHs) (**b**) in industrial effluents. The estrogenic compounds detected in the industrial effluents were 4-t-octylphenol (4-t-OP), 4-nonylphenols (4-NP), bisphenol-A (BPA), estrone (E1), and estradiol (E2). The PAH compounds detected in the industrial effluents were naphthalene (nap), acenaphthene (ace), fluorine (fl), phenanthrene (phe), anthracene (ant), fluoranthene (flu), pyrene (pyr), chrysene (chr), and benzo(a)pyrene (BaP). The horizontal lines represent the 5th, 50th, median, and 95th percentiles, and the boxes represent the 25th and 75th percentiles. Median and mean concentrations (ng/L) are displayed as solid and dashed horizontal lines, respectively. Outliers are shown as individual points. The percentage value below each box was the detection frequency of the compound.

surfactant degradation products, 4-t-OP and 4-NP were detected in the effluents from the textile and dyeing industry, with mean concentrations of 515 and 56,094 ng/L, respectively, which were much higher than concentrations originating from other industries (116 and 2,629 ng/L for the paper-making industry, 118 and 17,301 ng/L for the electronics industry, 3.1 and 1,310 ng/L for municipal wastewater plants, and 221 and 23,041 ng/L for the fine chemical industry, respectively).

Based on the chemical concentrations and their estrogenic equivalent factors, the EEQ of each effluent was calculated and listed in Table 2. For the effluent samples from the textile and dyeing plants and electronic and electroplate factories, 4-NP contributed more than 85% of the calculated EEQs. For the effluents from pulp and paper mills, the main EEQ contributors were 4-NP, E1, and E2 for some effluents with detection of E2, but primarily 4-NP (>60%) for those effluents without detection of E2. In the municipal effluents, both 4-NP and E1 accounted for most of the calculated EEQs; however, E2 was

Table 2. The measured concentrations and calculated estradiol equivalent concentrations (calculated EEQs) of estrogenic compounds in various effluents based on chemical analysis

		Compounds (ng/L)					
Effluent[a]	Samples	4-t-OP	4-NP	BPA	E1	E2	Calculated EEQ
TD	TD-1	96.9 ± 8.8[a]	38100 ± 10600	691 ± 93.1	ND	ND	24.2
	TD-2	60.6 ± 2.2	23000 ± 1040	522 ± 73.1	ND	ND	14.6
	TD-3	1701 ± 49	129000 ± 17500	1330 ± 52.6	1.6 ± 0.0	ND	83.6
	TD-4	713 ± 8.9	86600 ± 5300	474 ± 4.0	5.6 ± 0.1	ND	57.0
	TD-5	2.40 ± 1.5	3660 ± 2480	462 ± 49.0	<LOQ	ND	2.36
P	P-1	107 ± 1.2	1100 ± 89.6	4170 ± 158	9.1 ± 0.2	1.70 ± 0.3	5.63
	P-2	253 ± 13	4510 ± 98.3	598 ± 42.6	8.7 ± 5.9	1.70 ± 0.0	7.50
	P-3	235 ± 9.0	2960 ± 228	8970 ± 533	5.6 ± 2.1	1.60 ± 0.1	6.34
	P-4	7.90 ± 2.1	1090 ± 102	486 ± 59.4	1.00 ± 0.1	ND	1.05
	P-5	3.20 ± 2.0	1880 ± 308	746 ± 26.2	0.80 ± 0.1	ND	1.50
	P-6	72.1 ± 5.0	1760 ± 265	1660 ± 50.8	ND	<LOQ	1.36
	P-7	214 ± 17.9	6630 ± 459	980 ± 13.6	1.40 ± 0.2	ND	4.90
	P-8	37.2 ± 5.3	1106 ± 52.1	824 ± 32.9	ND	ND	0.82
E	E-1	ND	339 ± 9.1	217 ± 60.9	ND	ND	0.24
	E-2	321 ± 16.3	7330 ± 528	1040 ± 144	ND	ND	5.03
	E-3	93.7 ± 14.3	58100 ± 708	569 ± 8.3	ND	ND	36.8
	E-4	<LOQ	8060 ± 2530	496 ± 0.3	ND	ND	5.14
	E-5	38.6 ± 12.0	16500 ± 1210	568 ± 32.6	ND	ND	10.5
	E-6	19.4 ± 0.6	13500 ± 7010	976 ± 20.5	ND	ND	8.62
MW	MW-1	3.30 ± 1.1	1110 ± 306	367 ± 4.1	4.60 ± 0.2	<LOQ	2.11
	MW-2	2.5 ± 1.5	1030 ± 150	658 ± 5.5	5.20 ± 0.3	<LOQ	2.29
	MW-3	3.50 ± 0.5	2410 ± 263	751 ± 65.2	3.90 ± 0.5	<LOQ	2.76
	MW-4	<LOQ	691 ± 118	628 ± 13.5	ND	ND	0.50
FC	FC-1	2.40 ± 0.6	1230 ± 141	705 ± 75.5	3.00 ± 3.2	11.6 ± 0.0	13.4
	FC-2	32.1 ± 9.0	9430 ± 1910	549 ± 26.8	7.80 ± 6.0	1.80 ± 0.0	10.2
	FC-3	629 ± 116	58500 ± 37300	572 ± 62.2	21.9 ± 3.4	4.20 ± 0.3	48.3
LOQ (ng/L)		0.1	0.007	0.002	0.5	1	
Estrogenic equivalent factor[b]		0.00093	0.00063	0.00011	0.3	1	

[a] Mean ± standard deviation ($n = 2$).
[b] Zhao et al. [38].
TD = textile and dyeing plants; P = pulp and paper mills; E = electronic and electroplate factories; MW = municipal wastewater treatment plants; FC = fine chemical factories; 4-t-OP = 4-tert-octylphenol; 4-NP = 4-nonylphenol; BPA = bisphenol-A; TCS = triclosan; E1 = estrone; E2 = 17β-Estradiol; EEQ = estradiol equivalent concentrations; LOQ = limit of quantitation; ND = not detected.

below the quantification limit. Of the three effluent samples from fine chemical factories, E2 was responsible for nearly 90% of the calculated EEQ in one effluent sample, and 4-NP, E1, and E2 contributed almost all of the calculated estrogenic activities in the other two effluents. Thus, among the seven target estrogenic compounds, 4-NP contributed significantly to the estrogenic activities in most effluents from the Pearl River Delta region, although the calculated EEQs varied among the different types of effluents.

Polycyclic aromatic hydrocarbons in effluents

As shown in Figure 2, among the U.S. EPA 16 PAHs only nine compounds (naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, and BaP) were detected at concentrations of less than 200 ng/L in the effluents from the Pearl River Delta region (Table 3). Of the nine PAHs, five compounds (naphthalene, fluoroanthene, phenanthrene, pyrene, and chrysene) were frequently found in the effluents, with mean concentrations of 67, 51, 93, 46, and 49 ng/L, respectively. Two compounds, acenaphthene and BaP, were noticeably detected only in the effluent from an electronic and electroplate factory (Table 3).

The concentrations of PAHs in the effluents were converted into BaP EQs based on the toxic equivalency factors of all PAHs [48]. The calculated BaP EQs ranged from 0.36 to 47.5 ng/L (Table 3). Excluding the highest BaP EQ value, the average BaP EQ was only 0.78 ng/L in the effluents. The BaP EQs of all samples, except the sample E-2, were all far below 15 ng/L, which is the predicted no effect concentration of BaP for aquatic organisms reported by von der Ohe et al. [47]. Among the detected PAH compounds, chrysene contributed more than 50% of the total BaP EQs in most of the effluents.

In addition to the quantitative analysis of estrogenic compounds and PAHs, effluent extracts were also scanned by GC-MS to qualitatively analyze nontarget compounds. A GC-MS scan of the effluent extracts showed characteristic peaks of phthalates in almost all effluents, with their estimated concentrations of more than 1.0×10^4 ng/L. Meanwhile, characteristic peaks for 4-NP and 4-t-OP and their ethoxylates, as well as anilines and sulfamides, were identified in the effluents from textile and dyeing plants, electronic and electroplate factories, and fine chemical factories (See Supplemental Data). Characteristic peaks for BPA, sterols, and sterol derivatives were identified in the effluents from pulp and paper mills. These nontarget compounds also might contribute to the toxic effects in the industrial effluents.

DISCUSSION

Combined in vitro bioassays and chemical analysis demonstrated the presence of estrogenic activity, genotoxicity, and potential toxicants (five estrogenic compounds and nine PAHs) in the five types of effluents from the Pearl River Delta region. Correlation analysis of the measured and calculated EQs

Table 3. The measured concentrations and calculated BaP EQs of PAHs in various effluents based on chemical analysis

		Compounds (ng/L)									
Effluent	Samples	Nap	Ace	Fl	Phe	Ant	Flu	Pyr	Chr	BaP	Calculated BaP EQ
TD	TD-1	60.5 ± 14.7[a]	ND	54.8 ± 18.3	133 ± 41.7	ND	101 ± 20.7	64.5 ± 13.8	73.9 ± 17.5	ND	1.15
	TD-2	86 ± 35.3	ND	54.6 ± 11.9	135 ± 32.3	ND	ND	65.3 ± 7.4	75.8 ± 18.2	ND	1.1
	TD-3	87.6 ± 29.4	ND	59.0 ± 20.4	134 ± 50.4	ND	ND	51.0 ± 11.0	60.1 ± 14.0	ND	0.93
	TD-4	84.9 ± 18.1	ND	42.3 ± 13.2	102 ± 43.9	ND	ND	49.9 ± 14.4	60.8 ± 12.9	ND	0.89
	TD-5	89.9 ± 25.5	ND	47.5 ± 18.0	117 ± 34.5	ND	ND	57.2 ± 16.5	62.2 ± 7.6	ND	0.93
P	P-1	105 ± 31.2	ND	53.9 ± 22.2	101 ± 36.6	ND	ND	52.7 ± 22.7	62.0 ± 15.3	ND	0.93
	P-2	83.9 ± 36.5	ND	97.9 ± 24.6	182 ± 37.7	ND	ND	96.5 ± 25.6	121 ± 29.8	ND	1.68
	P-3	60.8 ± 1.8	ND	72.9 ± 22.1	118 ± 28.8	ND	ND	66.5 ± 22.6	72.5 ± 14.5	ND	1.04
	P-4	31.2 ± 9.8	ND	37.1 ± 5.2	70.1 ± 17.8	34.2 ± 14.8	13.0 ± 4.8	23.9 ± 2.5	26.9 ± 4.6	ND	0.79
	P-5	97 ± 22.5	ND	45.7 ± 4.6	115 ± 13.0	ND	<LOQ	55.6 ± 9.3	74.9 ± 17.8	ND	1.06
	P-6	90.5 ± 14.2	ND	47.3 ± 16.7	114 ± 35.7	ND	23.3 ± 6.2	56.0 ± 17.3	65.4 ± 10.1	ND	0.99
	P-7	109 ± 36.0	ND	48.9 ± 21.8	116 ± 42.6	ND	ND	61.5 ± 19.8	64.0 ± 15.5	ND	0.98
	P-8	63.4 ± 18.0	ND	66.0 ± 27.6	157 ± 46.3	ND	ND	77.3 ± 18.1	64.0 ± 14.7	ND	1
E	E-1	30.7 ± 14.9	ND	29.0 ± 0.1	48.9 ± 11.8	ND	ND	21.0 ± 5.2	32.6 ± 10.9	ND	0.46
	E-2	185 ± 31.7	153.5 ± 25.8	58.7 ± 36.6	38.8 ± 12.1	60.1 ± 12.0	ND	32.5 ± 3.5	33.6 ± 3.8	46.1 ± 26.6	47.5
	E-3	31.3 ± 14.4	ND	48.4 ± 3.0	87.4 ± 23.5	43.8 ± 22.1	21.9 ± 1.6	33.5 ± 12.9	32.6 ± 4.1	ND	0.99
	E-4	32.2 ± 18.9	ND	ND	76.4 ± 10.9	ND	ND	23.0 ± 5.2	33.0 ± 7.6	ND	0.46
	E-5	163 ± 51.0	ND	90.8 ± 38.0	ND	ND	ND	52.0 ± 12.0	36.9 ± 7.9	ND	0.67
	E-6	28.5 ± 16.3	ND	32.9 ± 9.9	29.9 ± 20.7	ND	ND	21.2 ± 8.5	25.6 ± 5.8	ND	0.37
MW	MW-1	39.0 ± 5.8	ND	39.5 ± 17.9	71.4 ± 29.8	ND	ND	25.6 ± 8.3	30.1 ± 6.8	ND	0.48
	MW-2	37.1 ± 9.6	ND	39.7 ± 7.9	80.2 ± 16.7	ND	ND	28.0 ± 3.2	34.1 ± 7.8	ND	0.53
	MW-3	27.6 ± 17.7	ND	30.5 ± 5.9	43.2 ± 4.9	ND	8.9 ± 2.0	18.5 ± 5.5	24.7 ± 2.8	ND	0.38
	MW-4	28.2 ± 15.1	ND	28.6 ± 11.6	48.4 ± 6.5	ND	9.7 ± 2.6	17.7 ± 5.8	22.7 ± 2.8	ND	0.36
FC	FC-1	29.7 ± 13.6	ND	ND	59.6 ± 6.1	ND	12.3 ± 4.3	19.7 ± 6.9	25.7 ± 5.7	ND	0.38
	FC-2	35.1 ± 19.9	ND	36.4 ± 3.2	65.7 ± 5.5	ND	19.8 ± 6.6	46.3 ± 19.4	31.2 ± 10.8	ND	0.52
	FC-3	28.4 ± 13.3	ND	ND	87.6 ± 17.8	ND	24.0 ± 7.8	79.1 ± 12.2	34.5 ± 9.7	ND	0.56
LOQ (ng/L)		0.5	1	1	0.4	0.2	0.1	0.2	0.2	0.1	
Relative potency[b]		0.001	0.001	0.001	0.001	0.01	0.001	0.001	0.01	1	

[a] mean ± STD (standard deviation) ($n = 2$).
[b] Petry et al. [48].
TD = textile and dyeing plants; P = pulp and paper mills; E = electronic and electroplate factories; MW = municipal wastewater treatment plants; FC = fine chemical factories; Nap = naphthalene; Ace = acenaphthene; Fl = fluorene; Phe = phenanthrene; Ant = anthraxcene; Flu = fluoranthene; Pyr = pyrene; Chr = chrysene; BaP = benzo[a]pyrene; EQ = equivalent concentration; LOQ = limit of quantitation; PAH = polycyclic aromatic hydrocarbon; ND = not detected.

was applied to analyze the cause–effect relationships. As shown in Figure 3, a good linear correlation was found between the measured EEQs and calculated EEQs ($r^2 = 0.9054$ and $p < 0.0001$), which indicates a high degree of consistency between the estrogenic activity and the presence of estrogenic compounds. However, from the slope of the modeling equation, we could see that the calculated EEQs from chemical analysis were higher than the measured EEQs by the YES bioassay. This suggests that some unknown compounds present in the effluents could have antagonistic action on the estrogenic activities of estrogenic compounds, which has been reported in the literature [49,50]. In fact, anti-estrogenic effects were observed in most industrial effluents (Table 1), which, to some extent, supports the viewpoint.

Anti-androgenic activities were also observed in the effluents from the Pearl River Delta region (Table 1), but no linear correlation was found between the measured EEQs and flutamide EQs for the industrial effluents ($r^2 = 0.082$ and $p = 0.1561$) (Fig. 3). This indicates that the chemicals with estrogenic activity contributed little to the anti-androgenic effects of those industrial effluents. The anti-androgenic effects of the industrial effluents might be caused by different contaminants. Similarly, because no androgenic effects were detected in all of the effluents, the contaminants that caused anti-estrogenic activities in the effluents did not result in any androgenic effects.

As for genotoxicity, a good relationship was found between the measured BaP EQs for indirect mutagenic effects and the measured 4-NQO EQs for direct mutagenic effects ($R^2 = 0.4689$ and $p = 0.002$) (Fig. 4). High consistency existed between direct mutagenic effects and indirect mutagenic effects for most effluents (Table 1). Therefore, most genotoxicants in the effluents could lead to mutagenic effects without metabolic activation.

Tables 1 and 3 show that the BaP EQs measured by the umu/SOS bioassay were approximately 1,000 times as high as the calculated BaP EQs based on PAH concentrations. Furthermore, no linear correlation was found between the measured BaP EQs and calculated BaP EQs ($r^2 = 0.0362$; $p = 0.4088$). These results from the present study suggest that some unknown compounds apart from PAHs were the toxicants that contributed to the genotoxicity of those effluents and that PAHs were only minor contributors.

A GC-MS scan of the effluent extracts showed a presence of many nontarget compounds such as nonylphenol ethoxylates, phthalates, and anilines. Some environmental estrogenic compounds including BPA, 4-NP, 4-t-OP, and their ethoxylates, have shown mutagenic effects in in vitro or in vivo assays [51–56]. Phthalate compounds, including dibutyl phthalate, diisobutyl phthalate, bis (2-ethylhexyl) phthalate, and mono-2-ethylhexyl phthalate, have potential mutagenic and carcinogenic effects on organisms and trick a possible pathway of tumor initiation in animals [57–61]. Aniline is nonmutagenic [62–64], but its derivates such as 2,4-dimethylaniline and 2,4,6-trimethylaniline were identified as mutagens and carcinogens

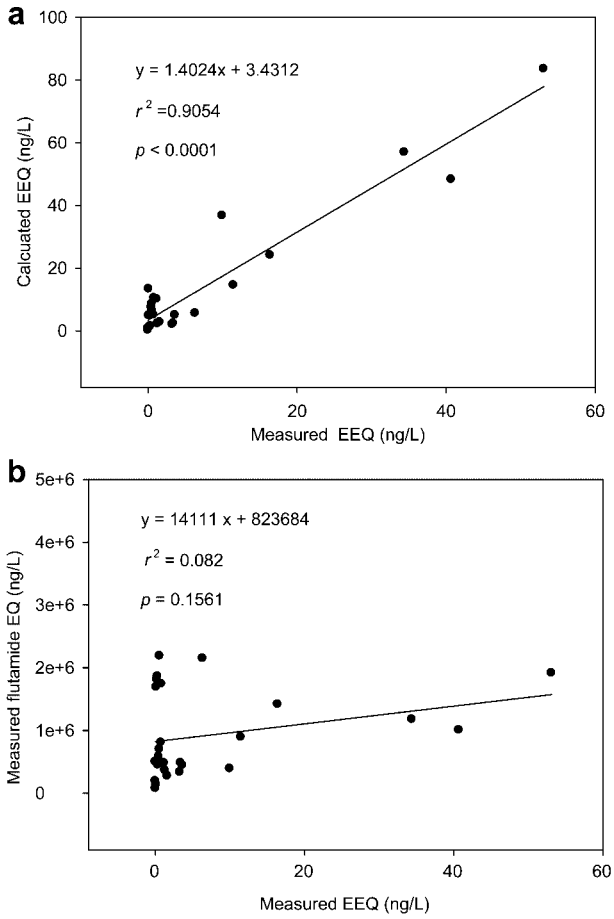

Fig. 3. Relationships between measured estrogenic activities (EEQs) and calculated EEQs in various effluents (**a**), and between measured estrogenic activities (EEQs) and measured anti-androgenic activities (flutamide EQs) in various effluents (**b**). EEQ = estradiol equivalent concentration.

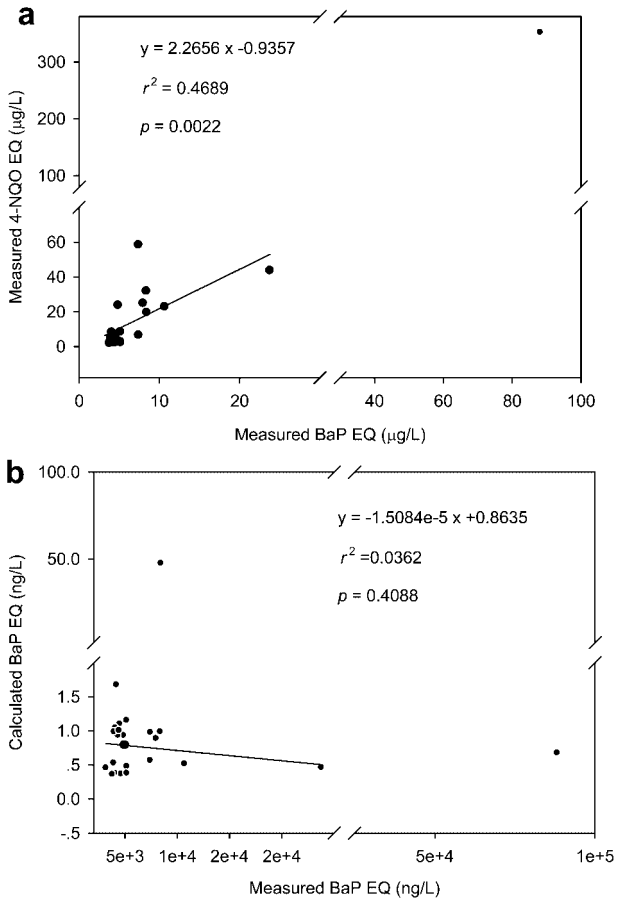

Fig. 4. Relationships between measured benzo[a]pyrene equivalent concentrations (BaP EQs) and 4-nitroquinoline-oxide equivalent concentrations (4-NQO EQs) in various effluents (**a**), and between measured BaP EQs and calculated BaP EQs in various effluents (**b**). In the umu/SOS assay without metabolic activation (–S9), the direct genotoxicity of an industrial effluent sample is expressed as a 4-NQO EQ, whereas with metabolic activation (+S9) the indirect genotoxicty is expressed as BaP EQ.

[65]. Aniline may react with norharman to form a mutagen (aminophenylnorharman) in organisms or in the presence of activation systems (S9 mix), which consequently causes genotoxicity [65–67]. Therefore, apart from the detected PAHs, other genotoxic compounds in the effluents led to the observed in vitro effects. However, further investigations are needed to understand more about these toxicants in industrial effluents.

Given the predicted no effect concentration values for estradiol and BaP of 1.5 ng/L and 15 ng/L, respectively [38,47], the risk quotients (ratio of the measured activity to the predicted no effect concentration) for some effluents would be higher than one. In the worst-case scenarios, without any dilution of these industrial effluents, potential high risks would be expected for aquatic organisms in the receiving environments. Therefore, better treatment technologies should be applied to give higher removals of these toxicants in these types of industrial wastewaters to reduce risks to the environment.

CONCLUSION

In vitro biological effects, including estrogenic, anti-estrogenic, and anti-androgenic activities, as well as genotoxicity, were detected in various industrial effluents in the Pearl River Delta region by using a series of in vitro bioassays. To the estrogenic activity in most of the industrial effluents, especially in those from textile and dyeing plants and electronic and electroplate factories, 4-NP was found to be the main contributor. The detected PAHs were only a minor contributor to the genotoxicity in the effluents; however, some nontarget compounds such as anilines could be responsible for the genotoxicity measured by the umu/SOS bioassay. A further in vivo study is required to understand the biological effects of these toxicants in industrial effluents. Owing to their high estrogenic and genotoxic risks, industrial effluents should be further treated before discharged into the receiving environments.

SUPPLEMENTAL DATA

Figs. S1–S6. (864 KB DOC).

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