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Occurrence and Potential Causes of Androgenic Activities in Source and Drinking Water in China

Xinxin Hu,[†,▽] Wei Shi,[†,▽,*] Si Wei,[†] Xiaowei Zhang,[†] Jianfang Feng,[†] Guanjiu Hu,[‡] Sulan Chen,[‡] John P. Giesy,[†,§,∥,⊥,#] and Hongxia Yu[†,*]

[†]State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing, People's Republic of China,

[‡]State Environmental Protection Key Laboratory of Monitoring and Analysis for Organic Pollutants in Surface Water, Jiangsu Provincial Environmental Monitoring Center, Nanjing, People's Republic of China,

[§]Department of Veterinary Biomedical Sciences and Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada,

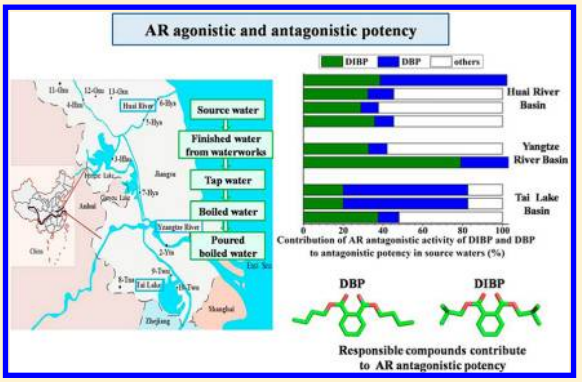
[∥]Department of Zoology, and Center for Integrative Toxicology, Michigan State University, East Lansing, MI, USA,

[⊥]School of Biological Sciences, University of Hong Kong, Hong Kong, SAR, People's Republic of China,

[#]Department of Biology and Chemistry and State Key Laboratory for Marine Pollution, City University of Hong Kong, Hong Kong, SAR, People's Republic of China

Supporting Information

ABSTRACT: The increased incidences of disorders of male reproductive tract as well as testicular and prostate cancers have been attributed to androgenic pollutants in the environment. Drinking water is one pathway of exposure through which humans can be exposed. In this study, both potencies of androgen receptor (AR) agonists and antagonists were determined in organic extracts of raw source water as well as finished water from waterworks, tap water, boiled water, and poured boiled water in eastern China. Ten of 13 samples of source water exhibited detectable AR antagonistic potencies with AR antagonist equivalents (Ant-AR-EQs) ranging from <15.3 (detection limit) to 140 μg flutamide/L. However, no AR agonistic activity was detected in any source water. All finished water from waterworks, tap water, boiled water, and poured boiled water exhibited neither AR agonistic nor antagonistic activity. Although potential risks are posed by source water, water treatment processes effectively removed AR antagonists. Boiling and pouring of water further removed these pollutants. Phthalate esters (PAEs) including diisobutyl phthalate (DIBP) and dibutyl phthalate (DBP) were identified as major contributors to AR antagonistic potencies in source waters. Metabolites of PAEs exhibited no AR antagonistic activity and did not increase potencies of PAEs when they coexist.



INTRODUCTION

In recent years, incidences of disorders of the male reproductive tract as well as testicular and prostate cancers have been reported to have increased.[1−3] These effects have been attributed to exposure to environmental contaminants through different pathways, and water drinking is one way people can be exposed to these contaminants. Previously, environment estrogens have been considered an important environmental factor associated with the increased risks of adverse outcomes.[4,5] However, large concentrations of androgens or androgen-like chemicals, such as dihydrotestosterone (DHT) and dibutyl phthalate (DBP), have also been found in surface waters used as drinking water at concentrations of 38.6 ± 12.6 ng/L and 15.6 ± 0.8 μg/L, respectively.[6,7] More recent studies have demonstrated that androgenic chemicals in the environment had the potential to disturb hormonal homeostasis and disrupt the development and function of male reproductive tract, such as feminization of males.[1,8,9] The widespread occurrence of androgenic chemicals in source and drinking water might also be an important factor contributing to adverse effects on development of the male reproductive system. Therefore, it was deemed to be essential to evaluate the androgenic contaminants in different phases of drinking water and the related androgenic effects posed to humans.

The Yangtze and Huai Rivers, Tai Lake and groundwater are the main sources of drinking water in eastern China. Detectable concentrations of organic pollutants including pesticides,

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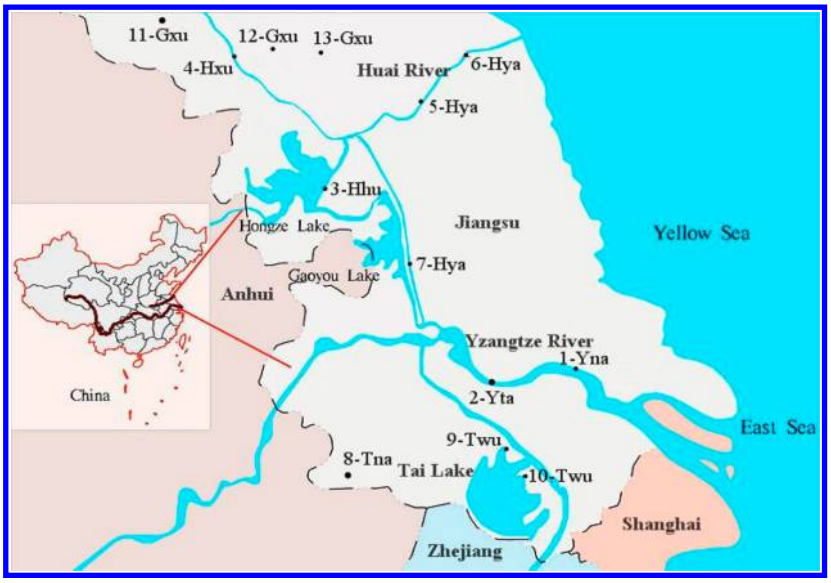

Figure 1. Map of the sampling locations in eastern China.

polycyclic aromatic hydrocarbons (PAHs) and phthalate esters have been found in these sources of drinking water.[10−12] Some of these detected chemicals have been reported to exhibit androgenic activity.[13−15] Moreover, it has been reported that the traditional water treatment processes in waterworks do not effectively remove these chemicals.[7,16] If present at sufficient concentrations in drinking water sources or drinking water, these chemicals might cause androgenic effects on residents in eastern China. Although advanced treatment processes, such as activated carbon adsorption, ultra filtration membrane, and magnetic ion-exchange resin, have been adapted in waterworks in eastern China, efficiencies of removal of androgenic contaminants and whether or not other transformation products were generated during conventional and advanced treatment processes were unknown. Chinese people usually drink boiled water, however, it was unclear if boiled water has any potential androgenic potency and androgenic chemicals. Moreover, some of these androgenic chemicals, such as phthalate esters, organochlorine pesticides (OCPs), and organophosphorus pesticides (OPs), can be transformed to corresponding metabolites.[17−19] Transformed compounds might pose risks to health and even increase androgenic potencies of their parent compounds when they coexist in mixtures. However, because there is little information available about their androgen receptor (AR) agonistic or antagonistic potencies, it was previously not possible to assess these risks.

Transactivation, reporter gene assays are useful, reliable, rapid, and reproducible in vitro methods to determine the endocrine disrupting potencies of single chemicals or mixtures.[13,14,20] The MDA-kb2 cell line is human breast cell line that is stably transfected with an androgen-responsive reporter gene.[21] This cell line has been used to screen for androgenic and antiandrogenic potencies of individual chemicals or mixtures collected from the environment.[22] In the present study, effect-directed analysis based on MDA-kb2 reporter gene assays and instrumental analysis was used to determine the AR agonistic and antagonistic potencies and to identify the responsible pollutants in source water or drinking water from eastern China. Specific objectives were to (1) examine the AR agonistic and antagonistic potencies of source water, finished water from waterworks, tap water, boiled water, and poured boiled water from different locations in eastern China; (2) identify chemicals responsible for AR agonistic and antagonistic potencies; (3) assess the potential health risks posed to aquatic organisms and humans.

MATERIALS AND METHODS

Chemicals and Reagents. Ten phthalate esters (PAEs) and six main metabolites of PAEs were used for reporter gene assays. Ten PAEs, 21 OPs, 25 OCPs, bisphenol A (BPA), nonyl phenol (NP), and octyl phenol (OP) were used for instrumental analysis. Information on these chemicals is provided in Table S1 in the Supporting Information (SI).

Sampling and Preparation. In August 2009, 13 samples of water were collected from drinking water sources including Yangtze River Basin, Huai River Basin, Tai Lake Basin, and groundwater in eastern China (Figure 1). Sites 1-Yna and 2-Yta were located in Yangtze River watershed. Sites 3-Hhu, 4-Hxu, 5-Hya, 6-Hya, and 7-Hya were located in Huai River watershed. Sites 8-Tna, 9-Twu, and 10-Twu were located in Tai Lake watershed. Samples collected from sites 11-Gxu, 12-Gxu, and 13-Gxu were groundwater. Source water samples were collected within five meters of intakes of waterworks. To determine the AR agonistic and antagonistic potencies in phases during drinking water processing and potential risk posed to residents, finished water from waterworks, tap water, boiled water and poured boiled water from the waterworks with the largest water supply and residences near sites 4-Hxu, 6-Hya, 9-Twu, and 10-Twu were also monitored. In waterworks near site 4-Hxu, activated carbon absorption is used. In waterworks near site 6-Hya, magnetic ion-exchange resin is used. In waterworks near sites 9-Twu and 10-Twu, ultra filtration membrane is used. Ten liters of water were collected in brown glass bottles at each location and transported immediately to the laboratory. Boiled water was the tap water which was boiled and cooled in a covered water boiler. Poured boiled water was boiled water which was poured into an open vessel fifty times and the open vessel then was sealed to cool the boiled water.

Samples of water were prepared by use of modifications of previously reported methods.[20,23] Briefly, analytes were separated and concentrated from water by solid phase extraction (SPE) with two tandem C18 cartridges (500 mg/6 mL, glass, Dikma Technology, China) which were preactivated

with different high-purity solvent including hexane (Merck, Darmstadt, Germany), dichloromethane (Tedia Co. Ltd., Fairfield, OH), acetone (Tedia Co. Ltd., Fairfield, OH), methanol (Tedia Co. Ltd., Fairfield, OH) and filtrated boiled milli-Q water. Approximately 2 L of water was passed through two tandem cartridges, and 10 cartridges were used for the water samples from one site ((10L/2L) × 2 cartridges = 10 cartridges). Analytes were eluted from cartridges successively with 10 mL hexane, 10 mL hexane and dichloromethane (4:1, v/v), and then 10 mL dichloromethane and methanol (1:1, v/v). Eluates from samples collected at each site were combined into a composite sample that was separated into two aliquots for use in bioassays and instrumental analyses, respectively. The aliquot for use in instrumental analysis was concentrated and blown to near dryness, and then reconstituted in 0.5 mL dichloromethane. The aliquot for use in the bioassay was concentrated, blown to near dryness and reconstituted in 0.2 mL dimethyl sulfoxide (DMSO, Tedia Co. Ltd., Fairfield, OH). The filtrated boiled milli-Q water used as procedure blanks was also extracted according to the procedure above-mentioned (Detailed information about procedure blanks is shown in SI). Extracts were stored at −20 °C.

MDA-kb2 Cell Culture and Reporter Gene Assay. MDA-kb2 cells (ATCC CRL-2713) were from a stock maintained at the Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada. Cells were maintained in L15 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen Corporation, Carlsbad, CA) at 37 °C in an ambient atmosphere. To minimize background activity in the bioassay, assay/exposure media was supplemented with 10% charcoal-dextran-stripped FBS (CDS-FBS, Biological Industries Ltd. Israel) instead of 10% FBS. Prior to bioassays, cells were cultured in assay media for at least 24 h and then plated at 1×10^5 cells/mL at a 384 well plate (Corning Inc., Corning, NY) with 80 μL of assay media per well and incubated 24 h at 37 °C, without CO_2. A series of concentrations of tested extracts (for detecting AR/GR (glucocorticoid receptor) agonistic activity) or supplemented with 1.0×10^{-9} mol/L DHT (for detecting AR antagonistic activity) were added into corresponding wells. Since both AR and GR are present in MDA-kb2 cells, both AR and GR agonistic activity might be expressed when MDA-kb2 cells were treated with compounds or environmental samples. Flutamide, a AR antagonist, was used to distinguish AR agonistic activity and GR agonistic activity. Blank and solvent control were also conducted in each plate. The final concentration of DMSO in medium did not exceed 0.1%. Various concentrations of DHT and flutamide were also included in each plate as positive controls for AR agonistic and antagonistic activity, respectively. After 24 h, exposure medium was removed from each well and 10 μL of 1 × lysis buffer (Promega, Madison, WI) was added per well. After 10 min for cell lysis, 25 μL of luciferase reagent was added per well and luminescence was quantified immediately in a Synergy H4 hybrid microplate reader (BioTek Instruments Inc., Winooski, VT). Before reporter gene assays were conducted, cytotoxicity of extracts alone or in presence of 1.0×10^{-9} mol/L DHT was examined by use of the MTT cytotoxicity test with previously reported methods.[24] Detailed information about cytotoxicity test is shown in SI.

Instrumental Analysis. First, suspected pollutants contributed to AR agonistic and antagonistic potencies in water samples including phthalate esters, pesticides and alkylphenols were qualitatively analyzed by use of GC-MS/MS (Thermo, San Jose, CA) in selected reaction monitoring (SRM) mode and LC-MS (Thermo, San Jose, CA) in SRM mode.

These compounds which were detectable in qualitative analyses were quantificationally measured. Phthalate esters were quantified by use of a Thermo TSQ Quantum Discovery triple-quadrupole mass spectrometer (San Jose, CA) that was operated in SRM mode. OCPs and OPs were detected using a Thermo Single Quadrupole GC-MS (San Jose, CA) in selected ion monitoring (SIM) mode. BPA, NP, and OP were quantified by use of RP-HPLC with a fluorescence and ultraviolet detector (Agilent Technologies, Palo Alto, CA). Mean recoveries and limits of quantification (LOQ) are given (SI Table S2). External standards were used to determine recoveries of detected compounds. Furthermore, internal standards including di-*n*-butyl phthalate-d4, bis(2-ethylhexyl)-Phthalate-3,4,5,6-d4, parathion-d_{10} and ^{13}C-PCB 141 were added to the tested extracts before instrumental analysis for quality control of phthalate esters, OPs and OCPs according to previously published methods.[25,26] More information on details of the instrumental analyses and quality assurance and quality control (QA/QC) is given in the SI.

Mass Balance Analysis. The AR agonistic and antagonistic equivalents (AR-EQs/Ant-AR-EQs) were calculated to measure the androgenic potencies and assess potential health risks of the tested water samples using modifications of previously described methods.[27,28] In the present study, none of the tested water samples exhibited AR agonistic activity, and therefore, the calculation of AR-EQs of water samples is shown in the SI.

Ant-AR-EQs of the tested water samples were derived by dividing the concentration of flutamide by enrichment factors of the tested samples that produced an equivalent inhibition (20%) of 1.0×10^{-9} mol/L DHT (eq 1).

$$\text{Ant-AR-EQ} = \frac{\text{EC}_{20}\ \text{of flutamide}}{\text{enrichment factors of tested samples}} \quad (1)$$

The relative potency to flutamide (ReP-flutamide) of individual chemicals was calculated as the ratio of the EC_{20}s respective for flutamide and tested chemicals (eq 2).

$$\text{ReP-flutamide} = \frac{\text{EC}_{20}\ \text{of flutamide}}{\text{EC}_{20}\ \text{of tested chemical}} \quad (2)$$

Predicted Ant-AR-EQs of chemicals were calculated by multiplying the RePs-flutamide by their concentrations in water samples. Total predicted Ant-AR-EQs of water samples were calculated as the sum of the predicted Ant-AR-EQs of the tested chemicals. The Ant-AR-EQ and total predicted Ant-AR-EQ of each sample were compared in a mass balance analysis to determine if the Ant-AR-EQs of the tested water samples have been accounted for by the total predicted Ant-AR-EQs based on all the detectable contaminants.

Data Analysis. All exposures were conducted in triplicate on each plate, and three experiments were performed for each tested sample. Values were expressed as mean ± standard deviations. Data were tested for by use of one-way analysis of variance (ANOVA) and Duncan's multiple comparisons test using SPSS 11 (SPSS Inc., Chicago, IL). Levels of significance were set as * ($p < 0.05$) and ** ($p < 0.01$). No significant difference was observed between field blanks and solvent controls in the bioassays. There was no significant difference among the three experiments.

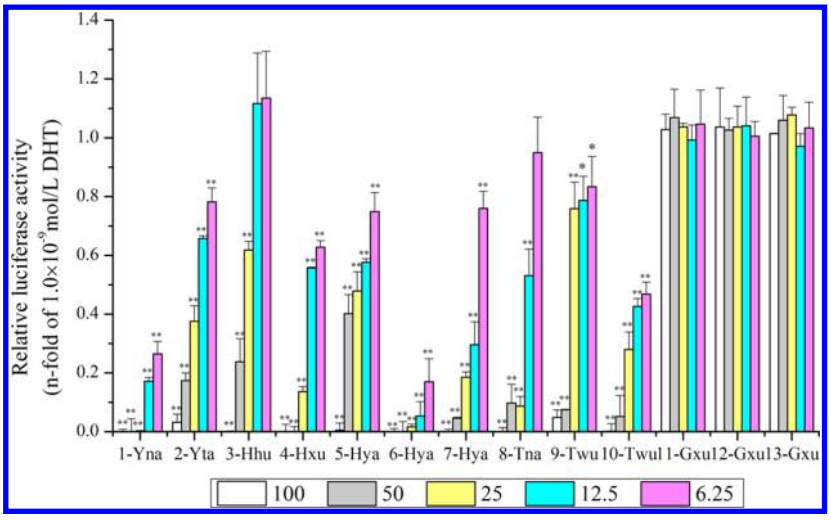

Figure 2. Androgen receptor (AR) antagonistic potency of source water measured by use of reporter gene assays based on MDA-kb2 cell at 100, 50, 25, 12.5, and 6.25 times the original concentration in water samples. The AR antagonistic effect of water samples was expressed as relative luciferase activity compared to 1.0×10^{-9} mol/L DHT. The levels of significance were set as ** ($p < 0.01$) and * ($p < 0.05$).

Table 1. AR Antagonistic Equivalents (Ant-AR-EQs) of Waters Based on Reporter Gene Assays and Predicted Ant-AR-EQs of Individual Chemicals Based on Instrumental Analysis

		predicted Ant-AR-EQs (μg flutamide/L)							
samples	Ant-AR-EQ (μg flutamide/L)	DBP	DIBP	DEP	DMP	BBP	DEHP	∑predicted Ant-AR-EQs (μg flutamide/L)	contribution of ∑predicted Ant-AR-EQs of phthalate esters to Ant-AR-EQ of water samples(%)
1-Yna	140 ± 13	13	46	0.0052	0.021	0.038	0.025	59	42
2-Yta	41 ± 2.7	10	32	0.0015	9.1×10^{-4}	0.077	0.010	42	104
3-Hhu	<15.3[a]	6.3	3.8	0.0044	0.0083	0.030	0.0083	10	103
4-Hxu	33 ± 1.5	4.3	11	0.0019	0.021	0.0046	0.0032	14	46
5-Hya	30 ± 2.9	2.7	8.7	0.0043	0.0066	0.0092	0.0027	11	38
6-Hya	43 ± 3.3	4.1	15	0.0063	0.0094	0.062	0.0050	20	46
7-Hya	41 ± 5.3	5.2	14	0.014	0.0066	0.028	0.0089	19	47
8-Tna	19 ± 2.7	2.0	6.9	6.2×10^{-4}	1.7×10^{-4}	0.020	0.0028	8.9	47
9-Twu	16 ± 1.1	10	3.2	0.012	0.0094	0.019	0.0096	13	83
10-Twu	76 ± 6.4	8.0	28	0.0043	0.0070	0.13	0.0096	37	48

[a]The detection limit of Ant-AR-EQ was 15.3 μg flutamide/L.

RESULTS AND DISCUSSION

Assay Validation. A high throughput 384-well microplate format was used in MDA-kb2 reporter gene assays for the first time. The reliability and sensitivity of the system were assessed in this study. The standard chemical DHT induced luciferase activity in a concentration-dependent manner in the range of 1.0×10^{-12} to 1.0×10^{-7} mol/L in MDA-kb2 reporter gene assays (Figure S1 in SI). Flutamide, a known AR antagonist, caused inhibition of the luciferase activity induced by 1.0×10^{-9} mol/L DHT in a concentration-dependent manner with the EC_{20} as 7.0×10^{-7} mol/L (Figure S2 in SI).

Androgenic Activity in Source Waters. None of the source water samples exhibited AR agonistic activity. It has been reported that main AR agonistic compounds were steroid hormones.[29] It indicated that concentrations of steroid hormones in these water samples were less than could be quantified by use of the bioassay. AR agonistic potencies in other environmental samples were also limited. All effluents from industries and municipal wastewater in China that were studied by Fang et al, exhibited no androgenic activity either.[30]

In the presence of 1.0×10^{-9} mol/L DHT, 10 of the 13 source water samples significantly suppressed expression of reporter gene in a concentration-dependent manner (Figure 2). At the maximal concentration (100 times the concentration in the source water), responses were significantly suppressed to less than 5% of the activity induced by 1.0×10^{-9} mol/L DHT at all the 10 sites. The Ant-AR-EQs for the 10 water samples ranged from <15.3 (detection limit) to 140 μg flutamide/L (Table 1), which were less than the results reported previously as 20.4−935 μg flutamide equivalent concentration (FEQ)/L in surface water from Pearl River system.[31] Flutamide can significantly reduce fecundity, embryo hatch and mature oocytes of female fathead minnow and cause spermatocyte necrosis and degeneration of male fishes at concentration of 651 μg/L in a 21 days exposure expriment.[32] The lowest observed effective concentration (LOEC) of flutamide to induce testis-ova in male medaka was 200 and 320 μg/L, respectively.[33,34] The Ant-AR-EQs in source water samples ranging from <15.3 (detection limit) to 140 μg flutamide/L were less than this reported LOEC for flutamide, however, by only a small margin of safety (200 μg/L/140 μg/L = 1.4). This indicates a potential risk to aquatic biota and warrants further attention. The greatest AR antagonistic activity was observed in water from location 1-Yna in the Yangtze River watershed.

Table 2. Concentrations of Chemicals (μg/L) in Source Water Samples from Eastern China

chemicals	1-Yna	2-Yta	3-Hhu	4-Hxu	5-Hya	6-Hya	7-Hya	8-Tna	9-Twu	10-Twu	11-Gxu	12-Gxu	13-Gxu
DBP	100	81	57	35	22	34	42	16	82	65	15	7.8	14
DIBP	200	140	17	47	39	68	61	31	14	130	15	8.3	11
DEP	0.29	0.084	0.25	0.11	0.24	0.35	0.76	0.030	0.65	0.24	0.12	0.073	0.091
DMP	1.2	0.054	0.49	1.3	0.39	0.56	0.40	0.010	0.56	0.42	0.012	0.011	0.016
BBP	0.98	2.0	0.78	0.12	0.24	1.6	0.73	0.51	0.48	3.3	0.068	0.14	0.18
DEHP	7.0	2.9	2.3	0.88	0.76	1.4	2.5	0.78	2.7	2.7	0.46	0.68	0.56
DNOP	2.9	2.0	3.7	1.2	3.5	2.5	6.8	2.6	1.7	3.1	0.44	3.2	0.54
DEHA	0.030	0.026	0.15	0.030	0.10	0.20	0.52	0.10	0.050	0.070	0.0079	0.056	0.0083
α-BHC	$<7.1 \times 10^{-4}$	0.001	$<7.1 \times 10^{-4}$	9.3×10^{-4}	$<7.1 \times 10^{-4}$	$<7.1 \times 10^{-4}$	0.001	9.0×10^{-4}	0.0018	7.5×10^{-4}	0.0015	0.0027	$<7.1 \times 10^{-4}$
hexachlorobenzene	$<6.6 \times 10^{-4}$	7.1×10^{-4}	$<6.6 \times 10^{-4}$	7.7×10^{-4}	6.6×10^{-4}	8.0×10^{-4}	0.0011	$<6.6 \times 10^{-4}$	0.0017	$<6.6 \times 10^{-4}$	0.001	0.0021	$<6.6 \times 10^{-4}$
β-BHC	$<6.9 \times 10^{-4}$	9.9×10^{-4}	$<6.9 \times 10^{-4}$	0.0011	8.2×10^{-4}	9.6×10^{-4}	0.002	0.0044	0.0031	0.0015	0.0022	0.0040	$<6.9 \times 10^{-4}$
γ-BHC	$<6.8 \times 10^{-4}$	6.8×10^{-4}	$<6.8 \times 10^{-4}$	0.001	$<6.8 \times 10^{-4}$	$<6.8 \times 10^{-4}$	0.0012	9.1×10^{-4}	0.0018	$<6.8 \times 10^{-4}$	0.0014	0.0025	$<6.8 \times 10^{-4}$
δ-BHC	$<6.7 \times 10^{-4}$	6.8×10^{-4}	$<6.7 \times 10^{-4}$	$<6.7 \times 10^{-4}$	$<6.7 \times 10^{-4}$	$<6.7 \times 10^{-4}$	$<6.7 \times 10^{-4}$	$<6.7 \times 10^{-4}$	$<6.7 \times 10^{-4}$	$<6.7 \times 10^{-4}$	$<6.7 \times 10^{-4}$	$<6.7 \times 10^{-4}$	$<6.7 \times 10^{-4}$
heptachlor	$<6.2 \times 10^{-4}$	$<6.2 \times 10^{-4}$	$<6.2 \times 10^{-4}$	$<6.2 \times 10^{-4}$	$<6.2 \times 10^{-4}$	$<6.2 \times 10^{-4}$	$<6.2 \times 10^{-4}$	$<6.2 \times 10^{-4}$	$<6.2 \times 10^{-4}$	$<6.2 \times 10^{-4}$	$<6.2 \times 10^{-4}$	$<6.2 \times 10^{-4}$	$<6.2 \times 10^{-4}$
aldrin	$<6.9 \times 10^{-4}$	$<6.9 \times 10^{-4}$	$<6.9 \times 10^{-4}$	$<6.9 \times 10^{-4}$	$<6.9 \times 10^{-4}$	$<6.9 \times 10^{-4}$	8.3×10^{-4}	$<6.9 \times 10^{-4}$	$<6.9 \times 10^{-4}$	$<6.9 \times 10^{-4}$	$<6.9 \times 10^{-4}$	$<6.9 \times 10^{-4}$	$<6.9 \times 10^{-4}$
heptachlor epoxide A	$<7.9 \times 10^{-4}$	$<7.9 \times 10^{-4}$	$<7.9 \times 10^{-4}$	$<7.9 \times 10^{-4}$	$<7.9 \times 10^{-4}$	$<7.9 \times 10^{-4}$	0.0051	$<7.9 \times 10^{-4}$	$<7.9 \times 10^{-4}$	$<7.9 \times 10^{-4}$	$<7.9 \times 10^{-4}$	$<7.9 \times 10^{-4}$	$<7.9 \times 10^{-4}$
endrin	$<9.2 \times 10^{-4}$	$<9.2 \times 10^{-4}$	$<9.2 \times 10^{-4}$	$<9.2 \times 10^{-4}$	$<9.2 \times 10^{-4}$	$<9.2 \times 10^{-4}$	0.0017	$<9.2 \times 10^{-4}$	$<9.2 \times 10^{-4}$	$<9.2 \times 10^{-4}$	$<9.2 \times 10^{-4}$	$<9.2 \times 10^{-4}$	$<9.2 \times 10^{-4}$
p,p′-DDD	$<7.0 \times 10^{-4}$	$<7.0 \times 10^{-4}$	$<7.0 \times 10^{-4}$	$<7.0 \times 10^{-4}$	$<7.0 \times 10^{-4}$	$<7.0 \times 10^{-4}$	2.4×10^{-4}	$<7.0 \times 10^{-4}$	$<7.0 \times 10^{-4}$	$<7.0 \times 10^{-4}$	$<7.0 \times 10^{-4}$	$<7.0 \times 10^{-4}$	$<7.0 \times 10^{-4}$
dichlorvos	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018	<0.0018	0.0030	<0.0018	<0.0018	<0.0018	<0.0018	0.0022	0.0017
mevinphos	<0.0022	<0.0022	<0.0022	0.0026	<0.0022	<0.0022	<0.0022	0.0059	<0.0022	<0.0022	<0.0022	<0.0022	<0.0022
demeton (a)	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	0.0057	<0.0013	<0.0013	<0.0013
ethoprop	<0.0012	<0.0012	<0.0012	<0.0012	0.0072	<0.0012	0.0074	<0.0012	<0.0012	<0.0012	<0.0012	<0.0012	<0.0012
naled	0.0035	0.0025	0.0016	0.0018	0.0089	0.0047	0.013	0.011	0.0019	0.0092	0.0019	0.0075	0.010
demeton (b)	0.0033	<0.0014	0.0017	<0.0014	<0.0014	<0.0014	<0.0014	<0.0014	<0.0014	<0.0014	<0.0014	<0.0014	<0.0014
diazinon	<0.0018	<0.0018	<0.0018	0.0030	<0.0018	0.0023	<0.0018	0.0063	<0.0018	0.0045	<0.0018	<0.0018	<0.0018
methyl parathion	0.010	0.012	0.0097	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013
ronnel	<0.0014	<0.0014	0.0023	<0.0014	0.0035	<0.0014	0.0021	<0.0014	<0.0014	<0.0014	<0.0014	<0.0014	<0.0014
fenthion	<0.0019	<0.0019	0.0046	0.023	0.013	0.011	<0.0019	<0.0019	<0.0019	0.037	<0.0019	0.0029	<0.0019
chlorpyrifos	0.0063	0.0053	0.0052	0.013	0.0087	0.011	0.0072	0.016	0.0086	0.021	0.0086	0.0021	0.0049
trichloronate	0.0054	<0.0018	<0.0018	<0.0018	0.0058	<0.0018	0.0025	0.0090	<0.0018	<0.0018	<0.0018	<0.0018	0.0034
merphos	<0.0017	<0.0017	<0.0017	<0.0017	0.0066	<0.0017	<0.0017	<0.0017	<0.0017	<0.0017	<0.0017	<0.0017	<0.0017
stirofos	0.020	0.0066	0.0075	0.019	0.0022	0.0072	0.0058	0.027	0.021	0.026	0.021	0.0047	0.0051
tokuthion	0.011	0.011	0.0059	0.0072	<0.0013	0.0071	0.0040	0.035	0.027	0.047	0.027	0.0036	0.0074
fensulfothion	0.013	0.0042	<0.0019	0.017	<0.0019	0.0057	0.0039	0.013	0.0049	0.026	0.0049	0.0025	0.0049
bolstar	<0.0014	<0.0014	<0.0014	<0.0014	<0.0014	0.0022	<0.0014	<0.0014	<0.0014	0.0048	<0.0014	<0.0014	<0.0014
OP	0.017	0.035	<0.011	<0.011	<0.011	0.013	0.046	0.037	0.017	0.021	<0.011	0.040	0.017
NP	0.041	0.043	0.014	0.020	0.020	0.014	0.045	0.041	0.022	0.038	0.013	0.065	0.022

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Several chemical industry parks were located at Naton City, and the industrial wastewater might be the main pollution sources at site 1-Yna. The Ant-AR-EQ of water sample at site 2-Yta from Yangtze River was also relatively great. These results indicated that the AR antagonistic potencies in the Yangtze River watershed were great and the pollution of the locations at the downstream was more serious than the upstream locations on the Yangtze River. Pollutants from the downstream of Yangtze River might pose risks to wildlife in the Yangtze River estuary and East Sea. None of the groundwater samples exhibited any AR antagonistic activity, which indicated that the groundwater in this region is relatively safe for use as source water.

Concentrations of AR Antagonists in Source Waters. In the qualitative analysis, 8 phthalate esters, 10 OCPs, 17 OPs, NP, and OP were detected. In order to determine their concentrations and their contributions to the AR antagonistic potencies in waters, they were further quantified (Table 2). Eight phthalate esters were detected in all 13 samples of water sources. Concentrations of diisobutyl phthalate (DIBP) and DBP were greater than those of other phthalate esters, ranging from 8.3 to 200 μg/L and from 7.8 to 100 μg/L, respectively. Concentrations of DBP in water were greater than the national standard for drinking water sources (3.0 μg/L) in China, which indicated that the pollution of DBP was serious and more measures should be taken for mitigation. Concentrations of DIBP were also great, however, there was no water quality standard for DIBP in China. Concentrations of DBP and DIBP were greatest in water from site 1-Yna, where greatest AR antagonistic activity was found. Moreover, concentrations of other phthalate esters were less than 5.0 μg/L at most sites.

Ten OCPs, 17 OPs, NP, and OP were detected (Table 2) in water samples. Concentrations of individual OCPs were all less than 0.0051 μg/L, a result that was consistent with previous results with the concentrations of individual OCPs ranging from 3.7×10^{-4} to 0.020 μg/L.[35] Concentrations of individual OPs ranged from ND to 0.047 μg/L, and concentrations of individual OPs among water samples exhibited no significant difference. NP and OP were detected in the samples of source waters with concentrations ranging from 0.013 to 0.065 and <0.011 to 0.046 μg/L, respectively.

Mass Balance Analysis. Concentrations of phthalate esters were several orders of magnitude greater than those of other pollutants (Table 2), which indicated that phthalate esters might be the major contributors to the AR antagonistic potencies in water samples. Therefore, their AR agonistic and antagonistic potencies were examined by use of reporter gene assays based on MDA-kb2 cell lines in the present study. None of the tested phthalate esters exhibited AR agonistic potency (data not shown), which was similar to what had been previously reported.[36]

DIBP, DBP, BBP, DMP, DEP, and DEHP exhibited significantly AR antagonistic potencies which were proportional to their concentrations (Figure 3), while DEHA and DNOP exhibited no AR antagonistic activity. DIBP exhibited cytotoxicity at concentrations greater than 1.0×10^{-4} mol/L in the presence of 1.0×10^{-9} mol/L DHT. Therefore, the maximal concentration in AR antagonistic activity determination of DIBP was 1.0×10^{-4} mol/L. EC_{20} values and the relative potencies to flutamide (RePs-flutamide) for these phthalate esters are shown in Table 3. DIBP caused the greatest inhibition of luciferase activity induced by 1.0×10^{-9} mol/L DHT at 1.0×10^{-4} mol/L and exhibited greater AR antagonistic

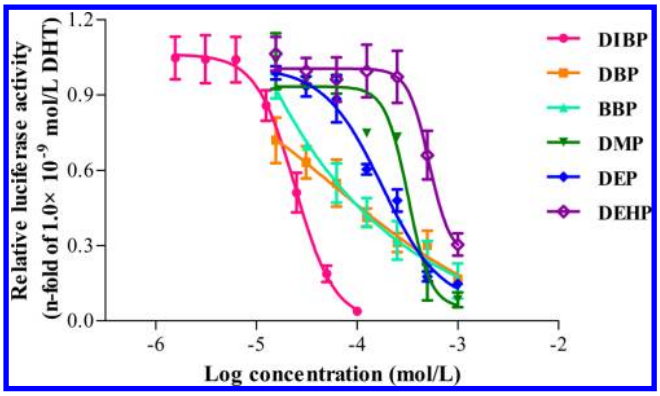

Figure 3. Androgen receptor (AR) antagonistic potencies of phthalate esters determined by use of MDA-kb2 cell reporter gene assays. The AR antagonistic potencies of phthalate esters were expressed as relative luciferase activity compared to 1.0×10^{-9} mol/L DHT.

Table 3. AR Antagonistic Potency (EC_{20}) and Relative Potencies to Flutamide (RePs-Flutamide) of Phthalate Esters[a]

chemicals	EC_{20} (mol/L)	RePs-flutamide
DBP	5.6×10^{-6}	0.12
DIBP	3.1×10^{-6}	0.23
BBP	1.6×10^{-5}	0.039
DEP	4.8×10^{-5}	0.018
DMP	5.9×10^{-5}	0.017
DEHP	1.4×10^{-4}	0.0036
DEHA	ND (>0.001)	–
DNOP	ND (>0.001)	–
DIDP	ND (>0.001)	–
DINP	ND (>0.001)	–

[a]ND: Not detected; –: Not applicable.

activity than other PAEs with the ReP-flutamide as 0.23. Previous studies which have examined the androgenic potencies of phthalate esters mainly focused on DBP, DEHP, BBP, and DEP. However, in the present study, DIBP exhibited the greatest AR antagonistic activity and concentrations of DIBP in water samples were greater than those of other phthalate esters. These results indicated that bioactivity of DIBP was greater. DBP also exhibited greater AR antagonistic activity than other PAEs with the ReP-flutamide as 0.12. DEHP caused less inhibition of luciferase activity induced by 1.0×10^{-9} mol/L DHT than other PAEs and the ReP-flutamide of DEHP was 0.0036, which was similar to results that reported that AR antagonistic potency of DEHP was less than that of DBP, BBP, or DEP.[36]

Predicted AR antagonistic potencies (predicted Ant-AR-EQs) of phthalate esters based on their individual RePs-flutamide and their concentrations in water are presented in Table 1. The relative potencies (RePs) of OCPs, OPs, NP, and OP ranged from 0.026 to 0.62 based on the antiandrogenic activity from previous reported literatures,[13,15,37−39] and no significant difference was found between RePs of these chemicals and RePs of phthalate esters. However, the concentrations of phthalate esters were 2−5 orders of magnitude greater than concentrations of OCPs, OPs, NP, and OP. Predicted Ant-AR-EQs of OCPs, OPs, NP, and OP were negligible. Predicted Ant-AR-EQs of DIBP were greatest and ranged from 3.2 to 46 μg flutamide/L. Predicted Ant-AR-EQs of DBP were also greater than other phthalate

esters and ranged from 2.0 to 13 μg flutamide/L. Predicted Ant-AR-EQs of DIBP and DBP at site 1-Yna from Yangtze River were greater than other sites.

Correlation analysis was used to detect the relationship between the Ant-AR-EQs of water samples and the total predicted Ant-AR-EQs of phthalate esters. The coefficient of determination (r^2) between the Ant-AR-EQs of water samples and predicted Ant-AR-EQs of phthalate esters was 0.802 (Figure 4). Total predicted Ant-AR-EQs of phthalate esters

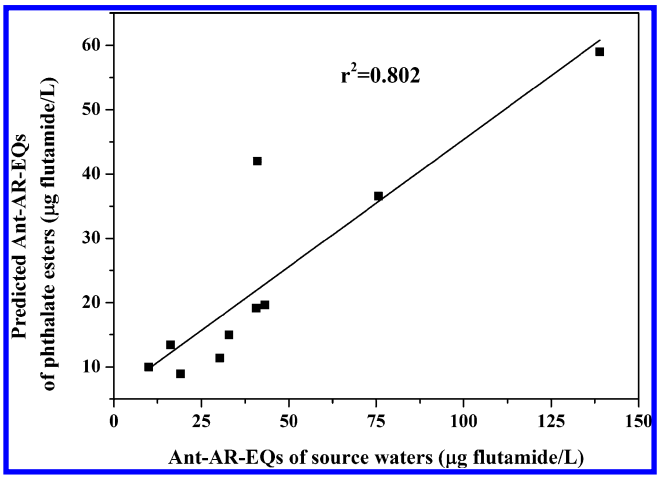

Figure 4. Correlation between AR antagonistic equivalents (Ant-AR-EQs) for source water samples based on MDA-kb2 cell reporter gene assays and predicted Ant-AR-EQs of phthalate esters based on concentrations in water samples.

accounted for 38−104% of the Ant-AR-EQs of water samples from different sites, with the greatest contribution at site 2-Yta from Yangtze River Basin. DIBP accounted for 20− 79% and DBP accounted for 8.9−64% of the Ant-AR-EQs. These results indicated that DIBP and DBP were responsible for the AR antagonistic potencies observed in source waters. DBP has been reported to adversely affect reproductive fitness and perinatal exposure to DBP could cause defectiveness and underdevelopment of epididymis, prostate, seminal vesicle, and other organs,[40,41] while studies of health risks of DIBP were fewer than those that have been done for DBP. Some countries and regions, such as China,[42] U.S.A.,[43] Australia,[44] and Canada,[45] have established surface water quality criteria for DBP. However, water quality criteria for DIBP have not been established by most countries. The USEPA has designated six phthalates, including DBP, BBP, DEP, DMP, DEHP, and DnOP as priority pollutants.[46] China has selected DMP, DBP, and DEHP as priority pollutants in water.[47] However, DIBP was not included in the list of priority pollutants by either USEPA or China. While more attention has been given to DBP than DIBP, production of DIBP in China was high and approximately 7.38×10^4 tons in 2007. In the present study, it was found that concentrations of DIBP in source waters from eastern China were relatively great. Moreover, other researchers have also found that concentrations of DIBP in indoor dust from buildings in Chinese cities were 5-fold greater than those from Albany, U.S.A.[48] It indicate that DIBP is a chemical of concern in China.

Further Risk Analysis. In order to evaluate the potential health risks to humans posed by AR antagonists through water drinking, removal efficiencies of AR antagonists in conventional processes associate with advanced water treatment processes (including activated carbon absorption, ultra filtration membrane, and magnetic ion-exchange resin) were determined. None of the finished water from waterworks exhibited AR agonistic or antagonistic activity, which illustrated that efficiencies of removal of AR antagonists through the related conventional and advanced water treatment processes were sufficient. Finished water from waterworks is routinely transported to residences, schools, hospitals, or other workplaces through pipelines. The AR agonistic and antagonistic potencies of tap water were measured to evaluate whether or not other AR agonists or antagonists were generated or entered into the water during the transportation with unplasticized polyvinyl chloride tube. None of the samples of tap water exhibited AR agonistic or antagonistic activity. Moreover, Chinese people usually drink boiled water. In order to know whether or not any AR agonists or antagonists could be generated or entered into water during boiling and pouring, the AR agonistic and antagonistic potencies of boiled water and poured boiled water were also determined. No AR agonistic or antagonistic potency was observed in boiled or poured water. These results are in agreement with the chemical data (Table 4), which predicted a maximum antagonist activity of 0.089 μg flutamide/L, which is much less than the method detection limit of 15.3 μg flutamide/L for the MDA-kb2 bioassay as used here.

Concentrations of phthalate esters in finished water from waterworks, tap water, and boiled water from four sites were determined by use of instrumental analysis (Table 4). Six phthalate esters were detected in finished water from waterworks with total concentrations of phthalate esters ranging from 250 to 640 ng/L. These concentrations of phthalate esters were less than those in untreated source waters. Efficiencies of removal of all the phthalate esters during drinking water treatments were greater than 84.6% (Table 4), especially for the removal efficiencies of DIBP and DBP (all above 99.7%). These results indicated that removal efficiencies of phthalate esters during drinking water treatment were effective. Six phthalate esters were detectable in most samples of tap water (Table 4) with total concentrations of phthalate esters ranging from 270 to 630 ng/L. Frequencies of detection of phthalate esters in boiled water were fewer than those in tap water, with total concentrations of phthalate esters ranging from 20 to 49 ng/L. Efficiencies of removal of these phthalate esters during boiling were more than 83.9% (Table 4). Since phthalate esters were not detected in water that was boiled and poured, this processes applied in individual homes can also remove residual phthalate esters.

It has been reported that phthalate esters are rapidly transformed to their corresponding metabolites in the human body, including monobutyl phthalate (MBP), monoethyl phthalate (MEP), monomethyl phthalate (MMP), mono-2-ethylhexyl phthalate (MEHP) and monobenzyl phthalate (MBzP), which have been commonly detected in human urine, breast milk, amniotic fluid, and umbilical cord blood.[17,49−51] However, androgenic activities of these metabolites were unknown. Therefore, the AR agonistic and antagonistic potencies of the major metabolites of the phthalate esters occurred in tap water were determined, including MBP, monoisobutyl phthalate (MIBP), MEP, MMP, MBzP, and MEHP. Combined AR agonistic and antagonistic potencies of these metabolites and their parent compounds were also determined (Table S3 in SI). None of the six metabolites exhibited AR agonistic or antagonistic activity. When MDA-kb2 cells were treated with

Table 4. Concentrations of Phthalate Esters in Finished Water from Waterworks, Tap Water, Boiled Water and Poured Boiled Water and the Predicted Ant-AR-EQs of Phthalate Esters in Different Water Samples

locations	Samples	DBP	DIBP	DEP	DMP	BBP	DEHP	∑predicted Ant-AR-EQs of phthalate esters (μg flutamide/L)
4-Hxu	finished water (μg/L)	0.10	0.097	0.012	0.057	0.0070	0.016	0.036
	tap water (μg/L)	0.087	0.076	0.0090	0.072	0.011	0.010	0.030
	boiled water (μg/L)	0.012	0.0083	<0.0013	<0.0013	<0.0013	<0.0030	0.003
	poured boiled water (μg/L)	<0.0020	$<3.0 \times 10^{-4}$	<0.0013	<0.0013	<0.0013	<0.0030	$<1.0 \times 10^{-4}$
	removal efficiency of drinking water treatment(%)	99.7	99.8	89.1	95.6	94.2	98.2	
	removal efficiency of boiling (%)	86.2	89.5	≈100	≈100	≈100	≈100	
6-Hya	finished water (μg/L)	0.072	0.11	0.034	0.086	0.0020	0.013	0.036
	tap water (μg/L)	0.068	0.087	0.054	0.10	<0.0013	0.022	0.031
	boiled water (μg/L)	0.0090	0.0014	<0.0013	0.0060	<0.0013	<0.0030	0.004
	poured boiled water (μg/L)	<0.0020	$<3.0 \times 10^{-4}$	<0.0013	<0.0013	<0.0013	<0.0030	$<1.0 \times 10^{-4}$
	removal efficiency of drinking water treatment(%)	99.8	99.8	90.3	84.6	99.9	99.1	
	removal efficiency of boiling (%)	86.8	83.9	≈100	94.1	≈100	≈100	
9-Twu	finished water (μg/L)	0.082	0.045	0.025	0.037	0.017	0.044	0.022
	tap water (μg/L)	0.17	0.057	0.033	0.024	0.013	0.061	0.036
	boiled water (μg/L)	0.011	0.0080	<0.0013	<0.0013	<0.0013	0.0060	0.003
	poured boiled water (μg/L)	<0.0020	$<3.0 \times 10^{-4}$	<0.0013	<0.0013	<0.0013	<0.0030	$<1.0 \times 10^{-4}$
	removal efficiency of drinking water treatment(%)	99.9	99.7	96.2	93.4	96.5	98.4	
	removal efficiency of boiling (%)	93.6	86.0	≈100	≈100	≈100	90.2	
10-Twu	finished water (μg/L)	0.19	0.28	0.034	0.028	0.057	0.049	0.089
	tap water (μg/L)	0.20	0.25	0.0056	0.031	0.046	0.038	0.086
	boiled water (μg/L)	0.012	0.024	0.0080	0.0030	0.0020	<0.0030	0.007
	poured boiled water (μg/L)	<0.0020	$<3.0 \times 10^{-4}$	<0.0013	<0.0013	<0.0013	<0.0030	$<1.0 \times 10^{-4}$
	removal efficiency of drinking water treatment(%)	99.7	99.8	85.8	93.3	98.3	98.2	
	removal efficiency of boiling (%)	94.2	90.6	85.7	90.3	95.7	≈100	

mixtures of phthalate esters and their corresponding metabolites, the AR agonistic and antagonistic potencies were not significantly changed relative to those of individual phthalate esters. Thus, the metabolites would not affect AR agonistic and antagonistic potencies of phthalate esters. Even so, it has been reported that these chemicals could cause toxic effect to wildlife or humans through other pathways. MBzP have been considered as teratogens in rats.[52] MBP and MBzP caused inhibition of proliferation in MCF-7 human breast cancer cell line, suggesting antiestrogenic activity.[53] Small concentrations of MEHP disrupted interactions between Sertoli cell and gonocyte and inhibited proliferation of Sertoli cell in rats.[54] Therefore, the potential health risks of these metabolites should not be ignored.

In conclusion, none of the source water samples from eastern China exhibited AR agonistic activity. All 10 of the surface water samples exhibited significant AR antagonistic potencies with Ant-AR-EQs ranging from <15.3 (detection limit) to 140 μg flutamide/L, and potential health risks might be posed to aquatic organisms and residents in this region. DIBP and DBP were identified the key chemicals responsible for the AR antagonistic potencies in source water, while contributions from OCPs, OPs, NP, and OP to AR antagonistic potencies were negligible. No AR agonistic and antagonistic activity was observed in any of the groundwater, or finished water from waterworks, tap water, boiled water or poured water. The conventional and advanced water treatment processes were effective in removing these pollutants with efficiencies greater than 84.6%. Boiling and pouring of water can further increase efficiencies of removal of these pollutants with the removal efficiencies above 83.9%. Although phthalate esters were detected in tap water and easily transformed to their corresponding metabolites in the human body, their metabolites exhibited neither AR agonistic nor antagonistic potency and did not increase the AR antagonistic potencies of parent phthalate esters when they coexist in mixtures. Because DIBP is frequently detected and has the greatest AR antagonistic effect, more attention should be paid to its toxic effects and the addition into the priority organic pollutants and emission standards in China for further management and control.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1 and S2, Tables S1, S2 and S3, the detailed information of chemicals and reagents, the detailed methods of sampling and preparation, cytotoxicity test, instrumental analysis and QA/QC, and the additional information of mass balance analysis and assay validation. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +86 25 8968 0356 (W.S.); +86 25 8968 0356 (H.Y.). Fax: +86 25 8968 0356 (W.S.); +86 25 8968 0356 (H.Y.). E-mail: njushiwei@nju.edu.cn (W.S.); yuhx@nju.edu.cn (H.Y.).

Author Contributions

▽X.H. and W.S. contributed equally to all aspects of conceptualizing, planning, method development, sample and data collection via bioassays and instrumental analyses and analysis and preparation of the manuscript.

Notes

The authors declare no competing financial interest.

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