Supporting Information

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

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AUTHOR CONTRIBUTIONS

▽ These authors 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.

Title:

Materials and Methods

Chemicals and reagents. Phthalate esters and dihydrotestosterone were purchased from Labor Dr. Ehrenstorfer-Schäfers (Augsburg, Germany). Metabolites of phthalate esters were obtained from AccuStandard Inc. (New Haven. CT. USA) or Toronto Research Chemicals Inc. (North York, ON. Canada). Pesticides OCPs and OPs were purchased from AccuStandard Inc. (New Haven. CT. USA) and Sigma (St. Louis, MO, USA), respectively. BPA, NP and OP were from Sigma. And the detailed information of these chemicals is listed in Table S1.

Sampling and preparation. In August, 2009, 13 samples of source water were collected from the main drinking water sources in eastern China including watersheds of the Yangtze and Huai Rivers, Tai Lake and groundwater (Figure 1). Moreover, finished waters were collected from the larger waterworks near sites 4-Hxu, 6-Hya, 9-Twu and 10-Twu, and tap waters were collected from residences near sites 4-Hxu, 6-Hya, 9-Twu and 10-Twu. In order to evaluate the potential risk to residents, boiled water and poured boiled water based on tap water from residences near sites 4-Hxu, 6-Hya, 9-Twu and 10-Twu were also collected. Boiled water was tap water which was boiled and cooled in a water boiler with cover. Poured boiled water was boiled water which was poured into an open vessel fifty times repeatedly and then the open vessel was sealed to cool the boiled water. At each location, ten liters of water were collected in brown glass bottles which were pre-cleaned with hexane (Merck, Darmstadt, Germany), dichloromethane (Tedia Co. Ltd, Fairfield, OH, USA), methanol (Tedia Co. Ltd, Fairfield, OH, USA) and milli-Q water. All the water samples were transported to laboratory immediately.

Cytotoxicity test. 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. Briefly, MDA-kb2 cells were seeded in 96-well plates at 1.0×10^{5} cells/mL and 100 μL assay medium was added into each well. Twenty-four

hours later, various concentrations of DHT and flutamide, different dilutions of tested extracts alone or with 1.0×10^{-9} mol/L DHT and 0.1% of DMSO were added into corresponding wells and incubated for another 24h. Subsequently, 25 μL of MTT (5 mg/mL in PBS) was added per well and incubated for 4h at 37 °C, without CO_2. Then 100 μL of DMSO was added to each well for 30 min to dissolve the crystals, and the absorbance was read at 570 nm using the Synergy H4 hybrid microplate reader.

Instrumental analysis. Chemicals of concern in water, including phthalate esters, organochlorine pesticides (OCPs), organophosphorus pesticides (OPs), bisphenol A (BPA), nonyl phenol (NP) and octyl phenol (OP) were quantified.

Quantitative analysis of phthalate esters were performed by use of a Thermo TSQ Quantum Discovery triple-quadrupole mass spectrometer (San Jose, CA, USA) in selected reaction monitoring (SRM) mode. Helium was used as carrier gas and flow rate was set at 1.0 mL/min. A pulsed splitless injector was used for injecting 1.5 μL of extract for analyzing phthalate esters. Temperature of the inlet was set as 250 °C. The initial oven temperature was set as 80 °C, held at 80 °C for 2 min, heated to 180 °C at 15 °C/min, held at 180 °C for 15 min, then heated to 300 °C at 15 °C/min, and held at 300 °C for 5 min.

Pesticides including OCPs and OPs were quantified by use of a Thermo Single Quadrupole GC-MS (San Jose, CA, USA) in selected ion monitoring (SIM) mode. Helium was used as carrier gas and flow rate was set at 1.0 mL/min. For analyzing OCPs, 1.5 μL of extract was injected into the column using a pulsed splitless injection. The oven temperature was set at 150 °C, heated to 290 °C at 4 °C/min, then heated to 310 °C at 15 °C/min, and held at 310 °C for 5 min. For analyzing OPs, 1.5 μL of extract was injected into the column using a pulsed splitless injection. The oven temperature was set at 50 °C, held at 50 °C for 1 min, heated to 100 °C at 10 °C/min, then heated to 280 °C at 7 °C/min, and held at 280 °C for 2 min.

BPA, NP and OP were quantified by use of reverse-phase high-performance liquid chromatography (RP-HPLC) (Agilent Technologies, Palo Alto, CA, USA). The methanol and water (1:4, v/v) was used as the mobile phase from 0 min to 5 min, and methanol was used as the mobile phase from 5 min to 11 min. The flow rate was set

as 1 mL/min. The temperature of column was set at 30 °C. A fluorescence and ultraviolet detector was used in detecting BPA, NP and OP.

Mass balance analysis. The AR agonistic equivalents (AR-EQs) of the tested water samples were calculated from the concentration of 20% of maximum activity of DHT (EC_{20}) and the enrichment factors of the tested source water which produced an equivalent expression (Equation 1).

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

QA/QC. During the process of instrumental analysis, 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. The regression coefficients (r^2) of calibration curves for all target analytes were higher than 0.99.

Blank runs of the chromatograph and direct injections of dichloromethane were made to check the presence of target compounds in the chromatographic system. None of the target compounds was present in the chromatograms. In the present study, procedural blank analyses were initially carried out with Milli-Q water, and the presence of phthalates was detected. Then the Milli-Q water was passed through solid phase extraction (SPE) with tandem C18 cartridges (500 mg/ 6mL, glass, Dikma Technology, China) which were pre-activated with different high-purity solvent including hexane (Merck, Darmstadt, Germany), dichloromethane (Tedia Co. Ltd, Fairfield, OH, USA), acetone (Tedia Co. Ltd, Fairfield, OH, USA) and methanol (Tedia Co. Ltd, Fairfield, OH, USA). The filtrated Milli-Q water was collected and further boiled and the related Milli-Q water was named as filtrated boiled Milli-Q water. Analyses of filtrated boiled Milli-Q water showed the presence of phthalates at very low levels and lower than those in our laboratory Milli-Q water. Because the unavailability of a perfect blank water sample, the filtrated boiled Milli-Q water was adopted for further performance studies. A procedural blank was performed for each batch of samples to check lab contamination. Concentrations of DBP, DIBP, BBP,

DEP, DMP, DEHP, DEHA, DNOP, DIDP and DINP in procedural blanks were 0.84±0.12, 0.18±0.01, 0.60±0.07, 0.74±0.06, 0.66±0.06, 1.20±0.18, 0.50±0.05, 0.34±0.04, 0.42±0.06 and 0.52±0.05 ng/L, respectively. For phthalate esters, limits of quantification (LOQ) for water samples were determined as ten times the standard deviation of the appropriate procedural blanks (Table S2). For OCPs, OPs, BPA, NP and OP, which were not detected in the procedural blanks, the LOQ was set at the laboratory LOQ which was ten times of S/N. The LOQ for each compound ranged from 0.30 to 28 ng/L (Table S2).

When sampling at each site, filtrated boiled Milli-Q water operated as the procedure of the sampling of water samples were used as the field blanks. Firstly, these field blanks were extracted using the same methods with water samples. Then they were detected by bioassays and instrumental analysis. All target analytes in field blank were below their corresponding LOQs. The final concentrations of compounds were that the concentrations in water samples subtracted concentrations in procedural blank. Because no AR ant/agonistic activity was found in extracts of field blanks and no Ant-AR-EQs were calculated, the data about AR ant/agonistic activity and Ant-AR-EQs of field blanks were not shown.

The procedural recovery and matrix spike recovery tests were conducted by spiking each target compounds into 10 L filtrated boiled Milli-Q water and 10 L water samples from source water, finished water, tap water, boiled water and poured boiled water, respectively. The spiked levels for phthalate esters were 1μg/L. The spiked levels were 5 ng/L and 10 ng/L for the pesticides and the phenols, respectively. Three replicates were conducted for QA/QC. The procedural recoveries and matrix spike recoveries of the tested compounds ranged from 82% to110% and 86% to 113%, respectively. The LOQs, procedural recoveries and matrix spike recoveries are shown in Table S2.

During sample analysis, quality control samples were consisted of duplicate samples, calibration check standards and solvent blanks. Duplicate samples were used to assure the precision and accuracy of each batch of samples, and the deviations of duplicate samples were less than 20%. Calibration check standards were run after

every ten sample to check the instrument. If the calibration check standards were out of ± 20% of its theoretical value based on the calibration curve, a new calibration curve was prepared. Solvent blanks were run prior to every ten sample to check the instrumental background.

Results and Discussion

Assay validation. In order to confirm reliability of reporter gene assays based on MDA-kb2 cell lines for detecting AR agonistic and antagonistic activities of the water samples or chemicals, the AR agonistic activity of DHT and AR antagonistic activity of flutamide were determined. DHT is a known AR agonist and flutamide is a known AR antagonist. DHT induced luciferase activity in a concentration-dependent manner in the range of 1.0×10^{-12} to 1.0×10^{-7} mol/L (Figure S1). The maximal induction of DHT was 11.8-fold of vehicle control at concentrations of 1.0×10^{-8} mol/L or greater. Flutamide significantly inhibited the luciferase activity which was induced by 1.0×10^{-9} mol DHT/L in a concentration-dependent manner in the range of 1.0×10^{-8} to 1.0×10^{-5} mol/L with the EC_{20} as 7.0×10^{-7} mol/L (Figure S2). Maximal inhibition was 78% at concentration of 1.0×10^{-5} mol/L of flutamide.

Figures.

Figure S1. The relative luciferase activity induced by dihydrotestosterone (DHT) in reporter gene assay based on MDA-kb2 cell lines.

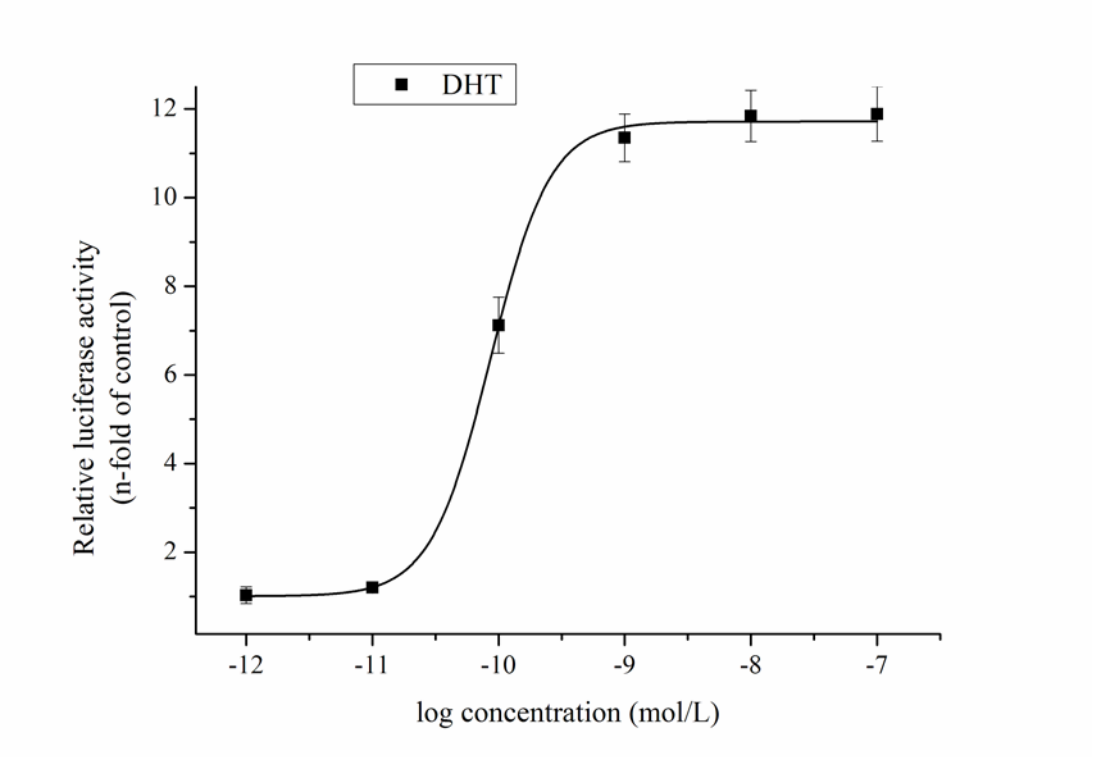
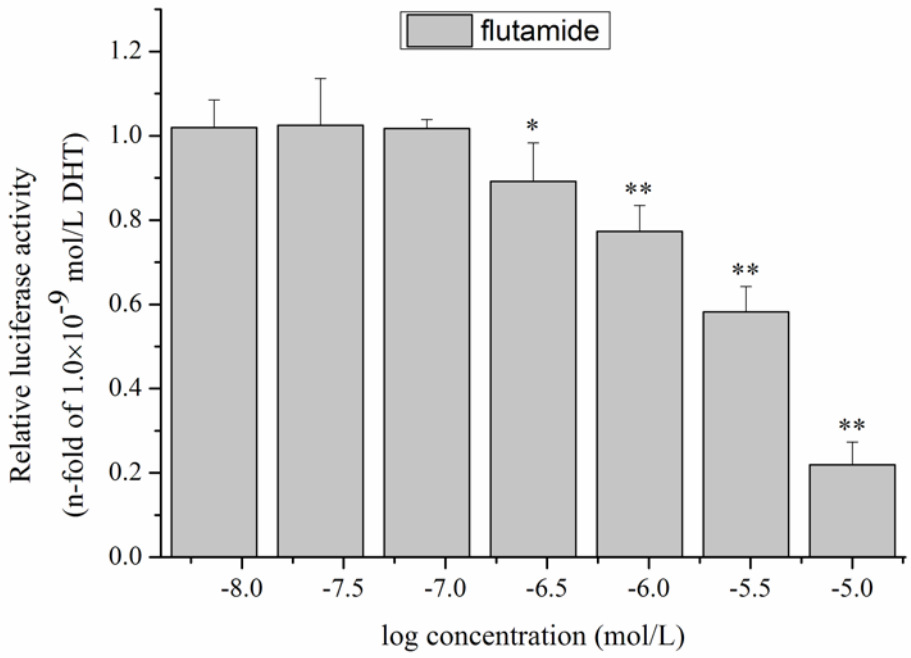


Figure S2. The relative luciferase activity comparing to 1.0×10^{-9} mol DHT/L treated with flutamide in reporter gene assay based on MDA-kb2 cell lines.



Tables.

Table S1. The information of the chemicals used in bioassays or instrumental analysis.

Chemicals	CAS no.	Abbreviation	Purity	Suppliers
dihydrotestosterone	521-18-6	DHT	>99.5%	Dr. Ehrenstorfer-Schäfers
Flutamide	13311-84-7		>99.0%	Sigma
Dibutyl phthalate	84-74-2	DBP	>99.0 %	Dr. Ehrenstorfer-Schäfers
Benzyl butyl phthalate	85-68-7	BBP	>99.5%	Dr. Ehrenstorfer-Schäfers
Diethyl phthalate	84-66-2	DEP	>99.5%	Dr. Ehrenstorfer-Schäfers
Dimethyl phthalate	131-11-3	DMP	>99.5%	Dr. Ehrenstorfer-Schäfers
Diisobutyl phthalate	84-69-5	DIBP	>99.0 %	Dr. Ehrenstorfer-Schäfers
Di-2-ethylhexyl phthalate	117-81-7	DEHP	>99.0 %	Dr. Ehrenstorfer-Schäfers
Diisononyl phthalate	28553-12-0	DINP	>99.0%	Dr. Ehrenstorfer-Schäfers
Diisodecyl phthalate	68515-48-0	DIDP	>99.5%	Dr. Ehrenstorfer-Schäfers
Bis(2-ethylhexyl) adipate	103-23-1	DEHA	>99.5%	Dr. Ehrenstorfer-Schäfers
Di-n-octyl phthalate	117-84-0	DNOP	>99.5%	Dr. Ehrenstorfer-Schäfers
Monobutyl phthalate	131-70-4	MNBP	>99.0%	AccuStandard
Monoisobutyl phthalate	30833-53-5	MIBP	>98.0%	TRC
Monobenzyl phthalate	2528-16-7	MBzP	>98.0%	AccuStandard
Monoethyl phthalate	2306-33-4	MEP	>98.0%	AccuStandard
Monomethyl phthalate	4376-18-5	MMP	>98.0%	AccuStandard
Mono-2-ethylhexyl phthalate	4376-20-9	MEHP	>90.0%	AccuStandard
Dichlorvos	62-73-7		99.8%	AccuStandard
Mevinphos	7786-34-7		99.8%	AccuStandard
Demeton-S	126-75-0		99.8%	AccuStandard

Ethoprop	13194-48-4	99.8%	AccuStandard
Naled	300-76-5	99.8%	AccuStandard
Phorate	298-02-2	99.8%	AccuStandard
Demeton-O	298-03-3	99.8%	AccuStandard
Diazinon	333-41-5	99.8%	AccuStandard
Disulfoton	298-04-4	99.8%	AccuStandard
Methyl parathion	298-00-0	99.8%	AccuStandard
Ronnel	299-84-3	99.8%	AccuStandard
Chlorpyrifos	2921-88-2	98.5%	AccuStandard
Fenthion	55-38-9	99.8%	AccuStandard
Trichloronate	327-98-0	99.8%	AccuStandard
Merphos	150-50-5	99.8%	AccuStandard
Stirofos	22248-79-9	99.8%	AccuStandard
Tokuthion	34643-46-4	99.8%	AccuStandard
Fensulfothion	115-90-2	99.8%	AccuStandard
Bolstar	35400-43-2	99.8%	AccuStandard
Coumaphos	56-72-4	99.8%	AccuStandard
Azinphos methyl	86-50-0	99.8%	AccuStandard
α-BHC	319-84-6	99.5%	Sigma
Hexachlorobenzene	118-74-1	99.5%	Sigma
β-BHC	319-85-7	99.5%	Sigma
γ-BHC	58-89-9	99.5%	Sigma
δ-BHC	319-86-8	99.5%	Sigma
Heptachlor	76-44-8	99.5%	Sigma
Aldrin	309-00-2	99.5%	Sigma
Isodrin	465-73-6	99.5%	Sigma
Heptachlor epoxide B	1024-57-3	99.5%	Sigma
Oxychlordane	26880-48-8	99.5%	Sigma
Heptachlor epoxide A	28044-83-9	99.5%	Sigma

γ-Chlordane	57-74-9		99.5%	Sigma
o,p'-DDE	3424-82-6		99.5%	Sigma
Endosulfan	959-98-8		99.5%	Sigma
α-Chlordane	5103-71-9		99.5%	Sigma
p,p'-DDE	72-55-9		99.5%	Sigma
Dieldrin	60-57-1		99.5%	Sigma
o,p'-DDD	53-19-0		99.5%	Sigma
Endrin	72-20-8		99.5%	Sigma
Endosulfan I	33213-65-9		99.5%	Sigma
o,p'-DDD	72-54-8		99.5%	Sigma
o,p'-DDT	789-02-6		99.5%	Sigma
p,p'-DDT	50-29-3		99.5%	Sigma
Methoxychlor	72-43-5		99.5%	Sigma
Mirex	2385-85-5		99.5%	Sigma
Bisphenol A	80-05-7	BPA	99.5%	Sigma
Nonyl phenol	25154-52-3	NP	99.5%	Sigma
Octylphenol	27193-28-8	OP	99.5%	Sigma

Dr. Ehrenstorfer-Schäfers : Dr. Ehrenstorfer-Schäfers, Augsburg, Germany; Sigma: Sigma, St. Louis, MO, USA; AccuStandard: AccuStandard Inc. New Haven. CT. USA; TRC: Toronto Research Chemicals Inc. North York, ON. Canada.

Table S2. Recoveries and limit of quantification (LOQ) for analytes.

	Chemicals	LOQ (ng/L)	Procedural recovery (n=3)		Matrix spike recovery (n=3)	
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Phthalate esters	DBP	2.0	97	4.2	99	5.7
	DIBP	0.30	89	6.7	97	5.3
	BBP	1.3	89	3.2	96	8.8
	DEP	1.3	84	1.3	90	4.3
	DMP	1.3	94	3.3	96	5.0
	DEHP	3.0	94	20	86	5.3
	DEHA	1.0	110	5.4	111	9.9
	DNOP	0.70	89	3.7	93	6.0
	DIDP	1.0	84	0.9	93	5.0
	DINP	1.0	85	3.7	90	3.3
Organophosphorus pesticides	Dichlorvos	1.8	110	1.7	98	2.1
	Mevinphos	2.2	84	13	88	7.0
	Demeton (a)	1.3	91	6.3	92	4.6
	Ethoprop	1.2	90	8.1	99	13.0
	Naled	1.5	89	8.9	101	10.3
	Phorate	1.2	89	8.9	103	11.3
	Demeton (b)	1.4	90	4.8	95	7.3
	Diazinon	1.8	93	2.1	98	5.4
	Disulfoton	1.8	91	8.2	99	11.6
	Methyl parathion	1.3	86	5.2	100	7.7
	Ronnel	1.4	86	5.7	94	8.3
	Chlorpyrifos	1.5	100	7.2	110	5.7
	Fenthion	1.9	91	3.2	103	8.7
	Trichloronate	1.8	93	11	106	9.3

	Merphos	1.7	92	6.2	103	11.6
	Stirofos	1.5	93	3.7	102	14.3
	Tokuthion	1.3	94	5.6	100	7.4
	Fensulfothion	1.9	82	0.97	101	13.5
	Bolstar	1.4	92	9.7	103	15.0
	Azinphos methyl	7.3	85	5.1	95	6.9
	Coumaphos	4.4	90	3.1	94	6.2
Organochlorine	α-BHC	0.71	90	4.5	99	10.3
pesticides	Hexachlorobenzene	0.66	90	2.3	95	8.6
	β-BHC	0.69	91	4.2	102	11.4
	γ-BHC	0.68	86	5.8	98	9.8
	δ-BHC	0.67	89	4.5	100	12.1
	Heptachlor	0.62	90	4.5	101	12.1
	Aldrin	0.69	93	6.4	101	11.7
	Isodrin	0.77	84	4.4	101	9.7
	Heptachlor epoxide B	0.75	88	5.1	97	11.1
	Oxychlordane	0.72	87	7.1	101	10.2
	Heptachlor epoxide A	0.79	91	7.4	108	8.2
	γ-Chlordane	1.5	90	8.5	98	10.3
	o,p’-DDE	0.81	93	5.3	102	11.2
	Endosulfan	1.0	89	3.0	100	12.9
	α-Chlordane	0.83	92	2.5	102	11.7
	p,p'-DDE	0.80	88	5.9	105	10.2
	Dieldrin	0.57	91	5.8	105	10.2
	o,p'-DDD	0.86	84	6.4	102	5.8
	Endrin	0.92	87	7.6	99	13.5
	Endosulfan I	0.68	97	1.2	110	9.9

	p,p'-DDD	0.70	91	9.0	105	8.5
	o,p'-DDT	0.62	98	3.4	109	7.5
	p,p'-DDT	0.60	86	2.5	104	10.0
	Methoxychlor	2.0	97	1.5	107	4.8
	Mirex	0.93	91	5.1	113	4.0
Other chemicals	BPA	28	88	4.0	101	6.8
	NP	11	83	2.0	102	7.9
	OP	12	90	5.0	112	4.5

Table S3. Phthalate esters and their corresponding metabolites.

Phthalate esters	Corresponding metabolites
DBP	MBP
DIBP	MIBP
DEP	MEP
DMP	MMP
BBP	MBzP
DEHP	MEHP