Estrogens in municipal wastewater and receiving waters in the Beijing-Tianjin-Hebei region, China: Occurrence and risk assessment of mixtures

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Abstract

The potentially high release of estrogens to surface waters as a result of high population density and local livestock production in the Beijing-Tianjin-Hebei region may pose adverse effects on the reproductive systems of aquatic organisms . This study found that total measured concentrations of estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethinylestradiol (EE2) and diethylstilbestrol (DES) were 468±27.2 ng/L in treated wastewater and 219±23 ng/L in river waters in this region. E2, E3 and EE2 were the predominant estrogens in river waters. The restriction of DES for human use should have been enforced, however concentrations of DES were relatively high compared to other studies. It has been estimated that the Haihe and Yongdingxin Rivers deliver approximately 1.8 tonnes of ∑estrogens to the Bohai Bay annually. Concentrations of individual estrogens were significantly higher in river waters in the dry season, however, mass loadings were significantly higher in the wet season. The E2-equivalent concentrations (EEQ) reached 1.2±0.16 and 0.64±0.08 μg-E2/L following long-term and short-term exposure estimates, respectively, in river waters with an average EE2 contribution of over 90%. This could potentially give rise to high risks to fish populations. The presence of estrogens in river waters largely derive from human excretion. Field studies on estrogenic effects on fish reproductive systems are required in this region as a result of high estrogen contamination levels.

Keywords: Estrogens; River waters; Wastewater; Environmental risks; Sources

1. Introduction

Over the past thirty years, natural and synthetic estrogens released to the environment have raised great scientific and regulatory interest, as a result of their global presence and potential disruption of the normal physiological functions of endocrine systems of wildlife and humans [1-5]. Natural estrogens such as estrone (E1), 17β-estradiol (E2) and estriol (E3), and synthetic estrogens such as 17α-ethinylestradiol (EE2) have been identified as the major contributors to endocrine disrupting activity in the aquatic environment [6]. The presence of intersex fishes induced by the presence of these estrogens has been widely reported throughout the world [4, 6, 7]. These chemicals can enter the aquatic environment via discharge from municipal wastewater treatment plants (WWTPs), point-source and non-point-source discharges of untreated domestic wastewaters, livestock farms, and land-applied biosolids. Although WWTPs can remove these chemicals relatively efficiently, low level discharge in WWTP effluents can potentially pose adverse reproductive effects in aquatic organisms [8, 9]. Therefore, continuous attention is required to quantify environmental releases, occurrence and risks of estrogens.

The Beijing-Tianjin-Hebei region has one of the highest population densities in China. The region also produced approximately 5.3 million tonnes of meat annually in 2016 and 2017 [10]. Therefore, large quantities of estrogens (e.g. E1, E2 and E3) could potentially be released from both human and animal excretion, and ultimately enter the Wenyu River, Beiyun River, Haihe River and Yongdingxin River running through Beijing, Tianjin and Langfang (in Hebei Province) through different pathways as

44 described above. Grill et al. (2018) suggested that EE2 use in Beijing is the highest
45 across China [11]. It is a principal component of oral contraceptives and also used in
46 hormone replacement therapy, so it enters the river systems mainly with domestic
47 wastewater. Previous estimates have identified E2 and EE2 as the most important
48 pharmaceuticals amongst several widely used drug classes entering the environment
49 potentially resulting in high risks in surface waters across China [12]. Their predicted
50 concentrations were elevated in the Beijing-Tianjin-Hebei region [11, 12], which may
51 also raise the issue of potential human health risks in this region [13-15].

52 Diethylstilbestrol (DES) is also a synthetic estrogen which has a long history of use. It
53 has been prescribed to pregnant women for prevention of miscarriages and other
54 pregnancy problems since 1938, but was banned by 1971 in the USA due to health risks
55 to pregnant women, as well as potential carcinogenicity or adverse effects to the
56 reproductive system of born of these pregnancies, who were exposed to DES before
57 birth [16, 17]. It is still occasionally used to treat advanced prostate cancer [18].
58 However, it is unclear when DES restrictions in China were introduced. Meanwhile,
59 DES has been used in feed supplements or in subcutaneous implants for livestock
60 production [19], which could lead to contamination in the environment as a result of
61 residues present in manure.

62 To the authors' best knowledge, only limited research has investigated the presence of
63 estrogens in untreated and treated wastewater in this region along with associated
64 environmental risks of estrogen mixtures. This study attempted to investigate the

65 sources and presence of estrogens in the Beijing-Tianjin-Hebei region and assess the
66 potential additive estrogenic mixture risks to fishes. The study also intended to
67 investigate whether DES could be detected in this region since its use has been restricted.
68 The study was designed to quantify mass fluxes and spatial and seasonal variations in
69 the study rivers relating these to potential sources. Such information is important to
70 develop local management plans to reduce environmental and potential human health
71 risks resulting from the release of these substances.

72 **2. Materials and methods**

73 **2.1 Chemicals**

74 The targeted chemicals for this study included three natural estrogens, i.e. E1, E2 and
75 E3, and two synthetic estrogens, i.e. DES and EE2, as mentioned above. Isotope-labeled
76 chemicals, including estrone-2,4,16,16-d_4 (E1-d_4), 17β-estradiol-2,4,16,16,17-d_5 (E2-
77 d_5), estriol-2,4-d_2 (E3-d_2), 17α-ethinylestradiol-2,4,16,16-d_4 (EE2-d_4), were used as
78 internal standards (ISs). All standards and the ISs were purchased from Sigma-Aldrich
79 (UK) with the purity ≥ 97%. The physicochemical properties of the selected estrogens
80 are provided in Table S1 in the Supporting Information (SI).

81 **2.2 Study area and sample collection**

82 Sampling campaigns were conducted during a dry season (October 2016) and during a
83 wet season (August 2017), during which no precipitation was recorded. However, there
84 were several heavy rainfall events before the sampling period in the wet season. The

85 Wenyu River, Beiyun River, Haihe River and Yongdingxin River constitute a sub-
86 catchment of the Haihe River catchment with a total population of more than 26 million.
87 The sub-catchment receives treated and untreated wastewater linked to 70% of
88 population and 90% of drainage tanks in the Beijing-Tianjin-Hebei region.

89 Municipal wastewater and receiving river waters in the Beijing-Tianjin-Hebei region
90 from 51 sampling sites were collected. To explore potential sources of estrogens to the
91 river systems, both treated and identified untreated wastewater (UW) samples were
92 collected. This included treated wastewater from the effluent of four major WWTPs in
93 Beijing (designed treatment capacity, 0.35 – 1 million m^3/day) and two major WWTPs
94 in Tianjin (0.15 and 0.4 million m^3/day). Details are given in Table S2. The four
95 WWTPs in Beijing serve a total of 6 million population and discharge to tributaries of
96 the Wenyu River within Beijing. The two WWTPs in Tianjin serve a population of
97 approximately 1 million and discharge into tributaries of the Yongdingxin River and
98 Haihe River, respectively, within Tianjin. UW samples were collected from three UW
99 discharge sites with unknown sources identified along the mainstream of the Wenyu
100 River. River water samples were taken from an additional 42 sampling sites along the
101 rivers receiving WWTP effluents, including the Wenyu (tributary, Lingou River),
102 Beiyun (Liangshui River), Haihe and Yongdingxin (Chaobaixin River) Rivers. The
103 sampling locations started from the outlet of the Shahe Reservoir in Beijing and ended
104 at the estuaries of the Haihe River and Yongdingxin River to the Baohai Bay (Fig. 1).

105 A total of 4 L water was taken at each sampling site. For river waters, the mixed 4 L

106 grab sample was taken from the middle and bank edge of rivers (0-20 cm below water
107 surface) at the cross section of each sampling site. The water samples from WWTP
108 effluents and UW discharges were collected from the outlets or sewers before
109 discharging into the rivers using a bucket. All water samples were stored in a pre-
110 cleaned amber glass bottle and acidified to pH=2.5 by using 2 mol/L hydrochloric acid
111 to stabilize the samples [20]. All samples were delivered to the laboratory on ice and
112 extracted within 24 h. The meteorological conditions and further details relating to the
113 sampling sites are provided in the SI (section S1.1).

114

115 **Fig. 1.** Location of the Haihe River sub-catchment with the distribution of sampling
116 sites

117 **2.3. Sample pretreatment and analysis**

118 Sample pretreatment was conducted according to methods in previously published

studies with minor modifications [20-22]. In brief, the water sample from each site was filtered through 0.7 μm glass fiber filters, split into triplicate samples (1 L each) and spiked with 100 ng ISs each. The filtered samples were extracted by the solid-phase extraction using HLB (hydrophilic−lipophilic-balanced) cartridges that were conditioned in sequence with the mixture (10 mL, 1:1, v/v) of acetonitrile (ACN) and ethyl acetate (EA), methanol (MeOH, 10 mL) and MQ water (10 mL) before extraction. Cartridges were eluted with 12 mL mixture of ACN and EA, followed by 12 mL MeOH after being loaded with water samples. The eluent was concentrated to 1 mL under a stream of nitrogen. The target estrogens were analyzed by ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). More detailed information on sample pretreatment, gradient program of LC separation and mass spectrometric parameters are described in SI (S1.2 and S1.3).

2.4 Quality assurance and quality control

The calibration curve for each target estrogen exhibited a strong linearity ($R^2 > 0.99$) over a wide range of concentrations (several to 500 or 1000 μg/L, equivalent to ng/L in water samples) (Table S4). The method quantification limit (MQL) for the selected estrogens ranged 0.47-6.35 ng/L (Table S4). For each batch of samples, reagent blanks, procedural blanks and sample replicates were analyzed to monitor possible contamination and instrumental performance. The experimental procedure was determined to be free of contamination. The relative standard deviations of the triplicate samples were less than 15%. The recoveries were determined using a standard addition

140 method at three spiking concentrations of 10, 50, 100 ng/L for river water and tap water
141 matrices. The absolute recoveries ranged72.6–93.9% in river waters and 82.4-105% in
142 tap water. More detailed information can be found in the section S1.4 and Table S4-S5
143 (SI).

144 **2.5 Environmental risk and linkage to effects to fish reproductive systems**

145 Environmental risks of mixtures of the five estrogens were assessed in this study. These
146 are major contributors to estrogenic activity in the aquatic environment, normally
147 exhibiting 3 to 7 orders of magnitude higher potencies compared to other endocrine
148 disrupting chemicals [23-26]. Typically, the estrogenic potency of estrogens is
149 measured in relation to E2, which has a defined potency of 1. The additive effects of
150 the five estrogens to aquatic species were compared to E2 equivalent (EEQ)
151 concentrations which were derived after accounting for the relative potencies of
152 individual estrogens, as described in Eq. 1 below.

153 $$EEQ = \sum_{i=1}^{n} C_i \times RPF_i \quad (1)$$

154 Where C_i and RPF_i refer to the measured concentration and relative potency factor
155 of estrogen i, respectively. As fish are likely to be the most sensitive aquatic taxa to
156 these estrogens and studies on estrogenic potencies and related RPF values were the
157 most abundant in fish among aquatic organisms for all the five estrogens [27, 28], this
158 study mainly focused on environmental risks to fish. As a reflection of environmental
159 risks to the whole river water system, indirect effects to other taxa via the food web
160 may result from changes in the fish population [28].

RPF values varied considerably in the literature especially for EE2, as a result of the selection of different fish test species, endpoints and assay methods. For example, the RPF of EE2 was found to be as high as 30.6 and 40 in inducing vitellogenin (VTG) in adult female zebrafish and adult male fathead minnows, respectively [29, 30], but only 5 in inducing intersex of fish in another study [31]. Jobling et al. used different sets of values to estimate EEQ for induction of intersex and VTG, respectively [4]. The range of RPF values for fish could range from 1.19 – 40 for EE2, 0.2 – 0.4 for E1 and 0.024 – 0.033 for E3 [4, 32-35]. In this study, RPF values of these estrogens were derived from the relative difference of PNECs (predicted no effect concentrations) protective of reproductive effects in fish following both long-term and short-term exposures [27, 34]. PNECs in the case of long-term exposures derived from species sensitivity distributions, and the corresponding RPF values were 0.33, 0.033 and 20 for E1, E3 and EE2; whilst PNECs for short-term exposures derived from no-observed-effect concentrations (NOECs), and the corresponding RPF values were 0.25, 0.025 and 10 for E1, E3 and EE2 [34]. RPF values for DES are very limited in the literature, however, 0.026, used in this study, was reported based on the median effective dose values [36].

3. Results and discussion

3.1 Estrogens in wastewater

Although surface runoff from soils contaminated by livestock waste can be an important diffuse input of steroidal estrogens to aquatic environment, WWTP effluents are considered dominant sources especially for heavily urbanized regions [37]. Estrogens

182 in effluents from conventional biological WWTPs can typically range from ng/L to
183 μg/L [38]. To identify potential sources in the highly urbanized region in this study,
184 estrogens in WWTP effluents and UW were analyzed. All five estrogens were detected
185 in 100% of samples collected from the effluents of the six WWTPs included in the study.
186 The average total concentration of ∑estrogens was 468±27.2 ng/L and the concentration
187 of individual estrogens ranged from 54.5 to 137 ng/L (Table 1). This was within the
188 concentration range for E1 (not detected (nd) – 205 ng/L) and E3 (nd – 590 ng/L), but
189 exceeding the range of E2 (nd – 44.6 ng/L) included in the global review by Liu et al
190 [39]. The estrogens found in the WWTP effluents were present at higher concentrations
191 than those from other studies. Previous studies have not always detected DES in WWTP
192 effluents in this area [40, 41], however, DES concentrations were relatively high in this
193 study with a detection rate of 100%. As abovementioned, DES is restricted for human
194 use but may still be used to treat livestock. In previous studies, E2, E3 and EE2 were
195 detected at concentrations below or around 10 ng/L in WWTP effluents in Beijing,
196 whilst E1 concentrations were much higher (> 80 ng/L) [40-42].

197 In the UW discharges, seasonal average concentrations were 94±22, 143±29, 133±41,
198 127±30 and 73±11 ng/L for E1, E2, E3, EE2 and DES, respectively. Concentrations
199 were significantly higher in WWTP effluents with the exception of EE2 and DES
200 (Tukey-Kramer HSD, $p < 0.05$). The predominant estrogens were E2, E3 and EE2 in
201 both WWTP effluents and UW discharges with a total contribution of approximately
202 70%. On average, EE2 was the most abundant in WWTP effluents (26%), although a
203 difference in composition existed between effluents in Beijing and Tianjin. In Beijing,

E2 exhibited the second highest proportion in effluents, however in Tianjin, E3 was the second most abundant, which, however, has a proportion very close to EE2. Previous studies have reported EE2 to be present at comparable concentrations to natural estrogens in WWTP effluents, although annual prescription rates are relatively low (e.g. 50 kg in Germany, production of 41 kg in China) [43, 44]. This could be a result of its higher stability and the cleavage of the principally excreted glucuronide conjugates [43]. In contrast to this study, E1 has been reported to be the most abundant in WWTP effluents, mostly due to its poor removal efficiency in WWTPs [40-42, 45]. However, E3 is the final estrogen human metabolite, and its presence in influents is likely to be high. E2 is the primary human metabolite. The excretion rates of the two estrogens from pregnant women is high with the excretion rate for E2 reaching up to 5 mg/day [38]. Therefore, these two estrogens could be more abundant in effluents than other estrogens. Different effluent compositions from separate studies and locations may be related to varying human excretion rates, population characteristics including number of pregnant women, chemical properties and numerous parameters that will affect their removal efficiencies in WWTPs, including temperature, flow rates and microbial activity [42, 46].

Table 1 Seasonal average concentrations of estrogens in WWTP effluents (mean ±STD, ng/L)

	E1	E2	E3	EE2	DES	Sum
WWTP1	74.7±32.2	125±24.3	112±26.0	137±9.3	64.8±19.5	513±51.9
WWTP2	78.8±20.3	111±21.6	95.6±6.6	118±13.2	54.5±16.7	458±64.8
WWTP3	78.5±39.8	114±24.3	93.8±2.7	116±6.8	61.7±23.4	464±75.3
WWTP4	84.9±36.7	101±19.7	106±14.9	119±7.1	70.4±21.1	481±73.0
WWTP5	67.0±21.8	93.8±7.2	117±16.9	118±12.8	66.6±21.8	462±31.6
WWTP6	58.8±21.9	99.6±13.3	107±4.5	110±13.0	56.0±24.5	432±73.4

Mean	73.8	108	105	120	62.3	468
STD	9.4	11.5	9.0	9.0	6.2	27.2

STD: standard deviation

3.2 Spatial distribution of estrogens concentration and composition in receiving rivers

The presence of estrogens in river water samples from Beijing to the estuaries entering Bohai Bay, receiving above municipal wastewater, were analyzed. In all river water samples, the average total concentration of ∑estrogens was 219 ± 23 ng/L, of which the three natural estrogens accounted for 62% averagely. The average concentrations were 33, 51, 53, 58 and 24 ng/L for E1, E2, E3, EE2 and DES, respectively. Comparing these data with other studies worldwide (Table S6), the concentrations of E1 were comparable with or lower than other studies; and the concentrations of the other four estrogens were relatively higher than those from other studies with the exception of data from 139 streams in the USA [47]. Lei et al. reported a lower concentration of all five estrogens in the Yongdingxin River compared to this study [48]. Overall, the concentrations reported in this study were higher than those from many other studies worldwide. This matches the estimate by previous studies in this region, which have suggested that emissions were high for four of the estrogens (except DES), even after WWTP removal, as above mentioned.

Fig. 2. Spatial distribution of average concentrations of ∑estrogens in the two seasons along rivers (A); River names are denoted in italic bold characters; Figure B is an enlargement showing the spatial distribution of the concentration within the dashed-line square in Figure A. The sites named UW1-3 are UW discharge sites; the sites marked with asterisk (*) indicate WWTPs.

The spatial distribution of average concentrations of ∑estrogens in the two seasons along the study rivers is illustrated in Fig. 2. It has been demonstrated that WWTP effluents and UW discharges were the major contributors of the estrogens measured in the study rivers, provided that estrogen concentrations in WWTP effluents or UWs were significantly higher than those in river waters (T-test, $p < 0.05$). Estrogen concentrations at the downstream site after the confluence of tributaries that receive WWTP effluents were generally higher than those at upstream sites (Figs. 2A and 2B). By using the Tukey-Kramer HSD test ($\alpha = 0.05$), ∑estrogens water concentrations were not significantly different between the four rivers. However, multifactor analysis of variance showed that individual estrogens may have exhibited significant concentration

differences between rivers. Specifically, concentrations of E1 were significantly higher in the Wenyu River compared to the other three rivers with no significant difference found among the other three rivers. Concentrations of EE2 were found to be significantly higher in the Yongdingxin River compared to the Wenyu River. Concentrations of DES were significantly higher in the Wenyu River than in the Beiyun and Haihe Rivers. No significant differences were found for the other individual estrogens among the other rivers not indicated above. If examining the difference between river reaches separated by administrative boundaries, it was found that concentrations of E1, DES and ∑estrogens in Beijing (site ID ≤ S23) were significantly higher than those in Langfang (with only three sites in) and Tianjin. This was probably a result of the higher population density in Beijing than in the other two cities. No significance was found between Beijing and the Tianjin-Langfang region for E2, E3 and EE2 individually.

The estrogen composition between the sampling campaigns was relatively stable along the four rivers with E2, E3 and EE2 being dominant especially for the Beiyun, Haihe and Yongdingxin rivers. Their concentrations were significantly higher than E1 and DES. Concentrations were not significantly different between E3 and E2, or between E3 and EE2, respectively; however, EE2 was found at significantly higher concentrations than E2. DES showed the lowest concentrations among the five estrogens which is reasonable given that its human use has been restricted.

3.3 Mass fluxes of estrogens along rivers and from WWTP effluents

River flow rates were estimated by measurements of the flow velocity (m/s) and the width and average depth of the river cross section at the sampling site when collecting samples. Average effluent flow rates were obtained from the WWTP managers. The mass fluxes of estrogens were therefore calculated by multiplying measured concentrations and flow rates. The mass flux of ∑estrogens from outlet of the Shahe Reservoir to the Wenyu River was estimated to be 6.3 g/h on average, with a total annual mass loading of ca. 55 kg. The seasonal average mass flux of ∑estrogens from the three UW discharging sites to rivers ranged from 2.4 to 3.6 g/h, with a total mass loading of approximately 81 kg/year, with natural estrogens accounting for ca. 65%. The mass flux of ∑estrogens was the highest from WWTP3 (19 g/h) which is the largest WWTP in Beijing with a 1 million m^3/day wastewater treatment capacity (Table S2). The total input of ∑estrogens from WWTPs 1-3 to the Wenyu River was estimated to be 303 kg/year with natural estrogens contributing approximately 62%. The mass flux of ∑estrogens in effluents from WWTP4 was estimated to be 12 g/h, with estimates of 2.9 g/h and 7.2 g/h for WWTP5 and WWTP6, respectively. The mass flux of ∑estrogens from one river to another and from tributaries to the mainstream is shown in Fig. 3. The estimated total mass loading of ∑estrogens was approximately 1.8 tonnes/year from the Haihe and Yongdingxin Rivers to the Bohai Bay, with natural estrogens contributing approximately 63.5%.

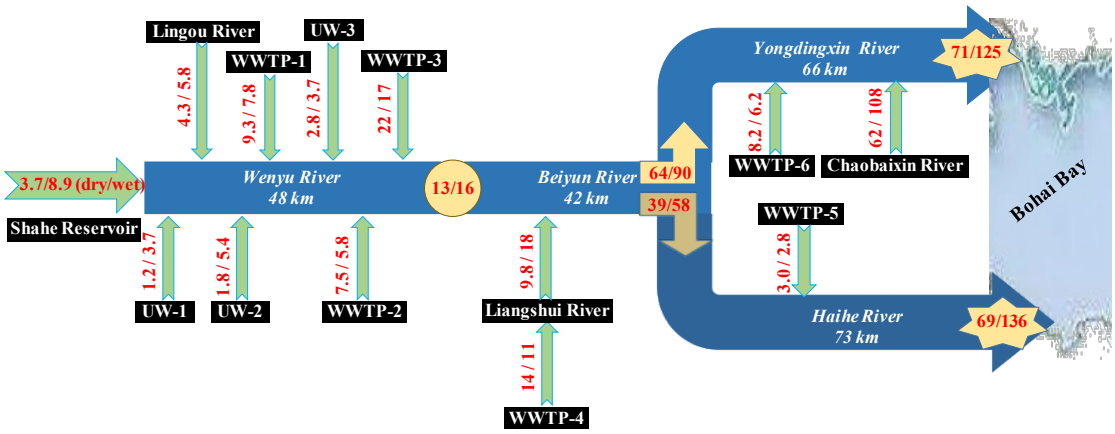

Fig. 3 Mass fluxes of ∑estrogens along rivers and mass loadings from WWTPs to rivers (g/h) for both seasons

3.4 Seasonal variation of concentrations and mass fluxes

Seasonal differences between the two sampling campaigns were investigated for both concentrations and mass fluxes. The average concentration of ∑estrogens in river waters was 248 ± 28.8 ng/L in the dry season and 190 ± 27.5 ng/L in the wet season (Table S7). Concentrations were significantly higher in the dry season for each individual estrogen (T-test, $p < 0.05$). In contrast, the mass flux of ∑estrogens in river waters was significantly higher in the wet season (averagely, 55.0 ± 45.4 g/h) than in the dry season (29.5 ± 25.6 g/h) (Table S8), with significant differences also found for individual estrogens (T-test, $p < 0.05$). This suggests that mass loadings were greater in the wet season although concentrations were diluted (Figs. 4 and S1). This matches previous findings for home and personal care product ingredients and antibiotics in the same rivers [22, 49]. These observations were probably a result of the combined effect of (1) higher dilution factors and (2) higher inputs of estrogens with sanitary sewer overflows and/or with land surface runoff during or after precipitation in the wet season compared to the dry season [12, 50, 51]. Land application of manure from livestock

operations followed by runoff could also be an important source of estrogens to the rivers in the wet season [52, 53]. Fig. S2 illustrates concentrations and mass fluxes of ∑estrogens, as well as for E1, E2 and DES individually in WWTP effluents, which demonstrates that they were significantly higher in the dry season compared to the wet season. This supports the probable occurrence of the sanitary sewer overflow and inputs with land surface runoff in the wet season, assuming that population sizes served by these WWTPs and human excretion rates were the same in the two seasons.

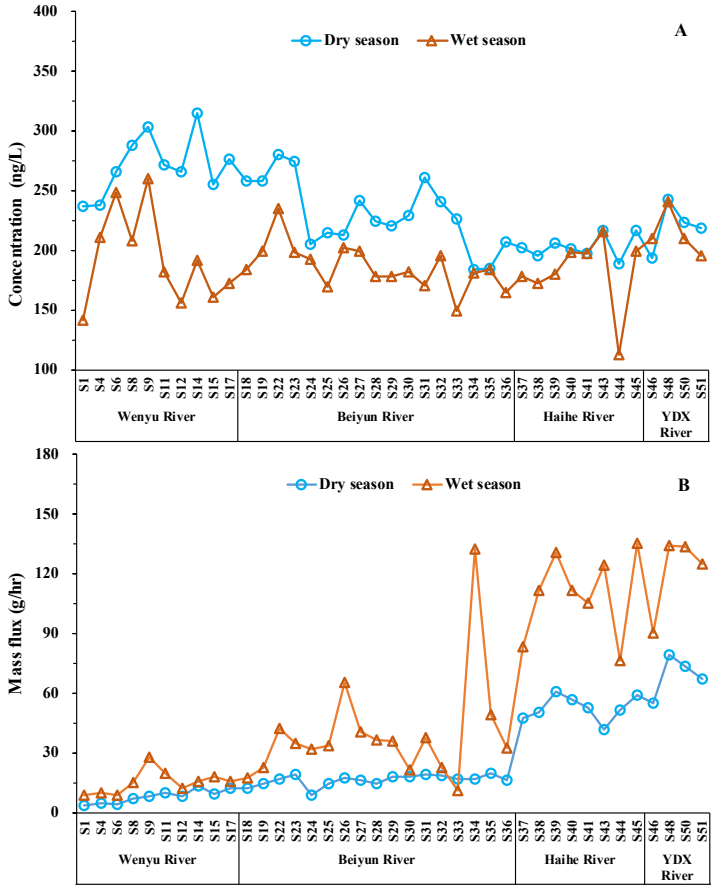

Fig. 4 Seasonal concentrations (A) and mass fluxes (B) of ∑estrogens

A comparison of estrogen composition in treated and untreated wastewaters and river waters between the two sampling campaigns was carried out. The percentage contribution from each estrogen was transformed (arcsine squareroot) before a

statistical comparison using the T-test ($\alpha = 0.05$). River water samples across the whole target region showed significantly higher proportion of E1 and DES in the dry season compared to the wet season, with an inverse pattern observed for E2, E3 and EE2. This seasonal feature generally matched those for UW and WWTP effluents, with the exception for E2, for which the seasonal difference in the composition was not significant in both UW and WWTP effluents (Fig. S3). However, across the study rivers (Fig. 5) the proportion of EE2 was not significantly different between the two seasons in the Wenyu and Haihe Rivers. This was also the case for the proportion of E3 in the Haihe and Yongdingxin rivers. Additionally, seasonal differences were insignificant for E1 and DES proportions in the Haihe River, and for E2 proportions in Yongdingxin River. The seasonal composition pattern for the other estrogens across the other rivers aligned with the pattern in river waters for the whole region.

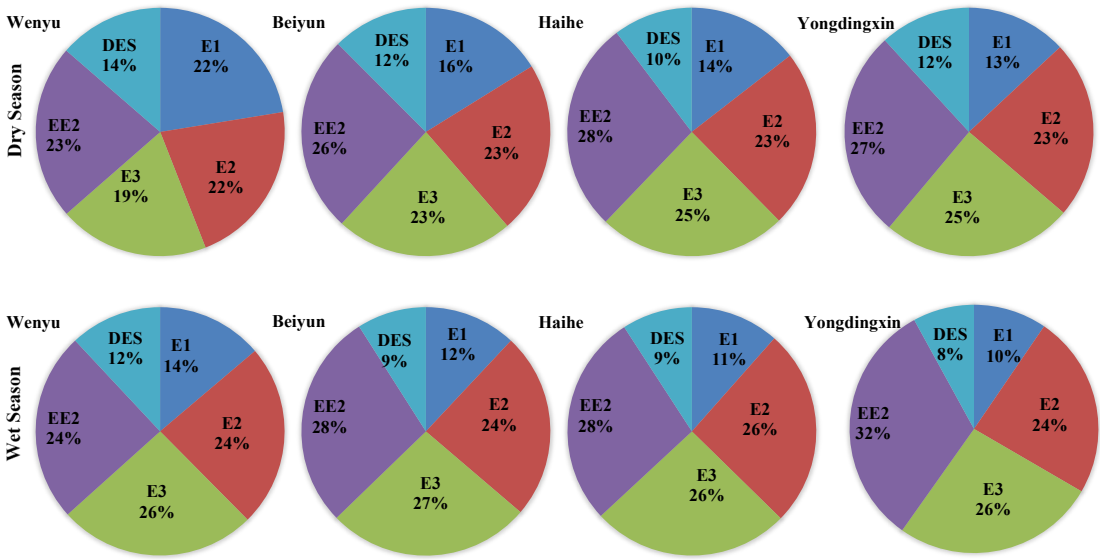

Fig. 5 Composition of estrogens in river waters of different river reaches in the dry and wet seasons

3.5 Risk assessment of estrogen mixtures

Adverse effects and associated risks were assessed for the mixture of estrogens rather than for individual substances as a result of multicomponent chemical "cocktail" in reality. The EEQ for the five measured estrogens was calculated to provide an assessment of risks to fish induced by the mixture of estrogens. RPF values from both long-term and short-term fish studies were taken from the literature. The corresponding EEQ was compared with the long-term E2 PNEC (2 ng/L) and the short-term E2 PNEC (5 ng/L), respectively [27]. The estimated additive EEQ was 1.2±0.16 μg-E2/L (range, 0.96 – 1.7 μg-E2/L) following long-term exposures and 0.64±0.08 μg-E2/L (range, 0.5 – 0.86 μg-E2/L) following short-term exposures in river waters using averaged data. The average additive EEQ was 2.5 μg-E2/L and 2.7 μg-E2/L in WWTP effluents and UW, respectively, following long-term exposures; and 1.3 and 1.4 μg-E2/L in WWTP effluents and UW, respectively, following short-term exposures. The data clearly shows that the EEQ at all sampling sites exceed both the long-term and short-term E2 PNECs, which indicated a high risk to fish from exposure to estrogen mixtures in these rivers. Additionally, the estimated EEQ was higher than the NOECs for inducing intersex (1 ng/L) and VTG (5 ng/L) and even higher than the LOECs (the lowest observable effect concentrations) for inducing intersex (10 ng/L) and VTG (25 ng/L) selected by Jobling et al. for their risk assessment [4]. Arlos et al. estimated that an EEQ ≥10 ng-E2/L was associated with high intersex incidence and severity [54]. Therefore, concentrations of estrogens measured in this study in the target region are likely to have been high enough to cause intersex or VTG etc., which will affect the reproduction and ultimately the population size of fishes locally. Subsequently, the abundance of other aquatic taxa,

366 such as algae, zooplankton, microorganism and invertebrates, might be affected because
367 of trophic linkage to fish [28]. Field observation of feminization, intersex or synthesis
368 of plasma VTG in wild fish induced by estrogens, effects on fish populations and other
369 aquatic taxa communities has been widely reported in the UK and the USA [4, 6, 55,
370 56], but is extremely rare in China.

371 The percentage of EEQ from individual estrogens could reflect the contribution of each
372 estrogen to risks induced by mixtures. Fig. 6 illustrates that EE2 was the major
373 contributor (>90%) of the cumulative estrogenic activity to fish in the target region. The
374 percentage was similar in municipal wastewater and river waters receiving the
375 wastewater as shown in Fig. 6. This is in contrast with the chemical mass compositions
376 in river waters and wastewaters (Figs. 5 and S3), which suggests that concentrations
377 alone do not reflect the environmental risk. As a result, estrogenic potency should be
378 considered in addition to concentration profiles. As EE2 contributed to higher potency
379 in long-term exposures (a selected RPF of 20 in this study) than in short-term exposures
380 (a selected RPF of 10), the percentage of its EEQ was higher in the case of long-term
381 exposures (95%) than short-term exposures (90-91%). E2 followed EE2 and showed
382 the second highest contribution to EEQ, ranging from 4.2 to 4.3% following long-term
383 exposures and 8.0-8.1% following short-term exposures. This observation aligns with
384 those in river waters of eight Asian countries including China reported by Duong et al.
385 [57]. They found E2 and EE2 made a predominant contribution toward estrogenic
386 activity. DES shared the lowest percentage contribution to EEQ with the lowest mass
387 concentrations and relatively lower RPF values compared to the other estrogens.

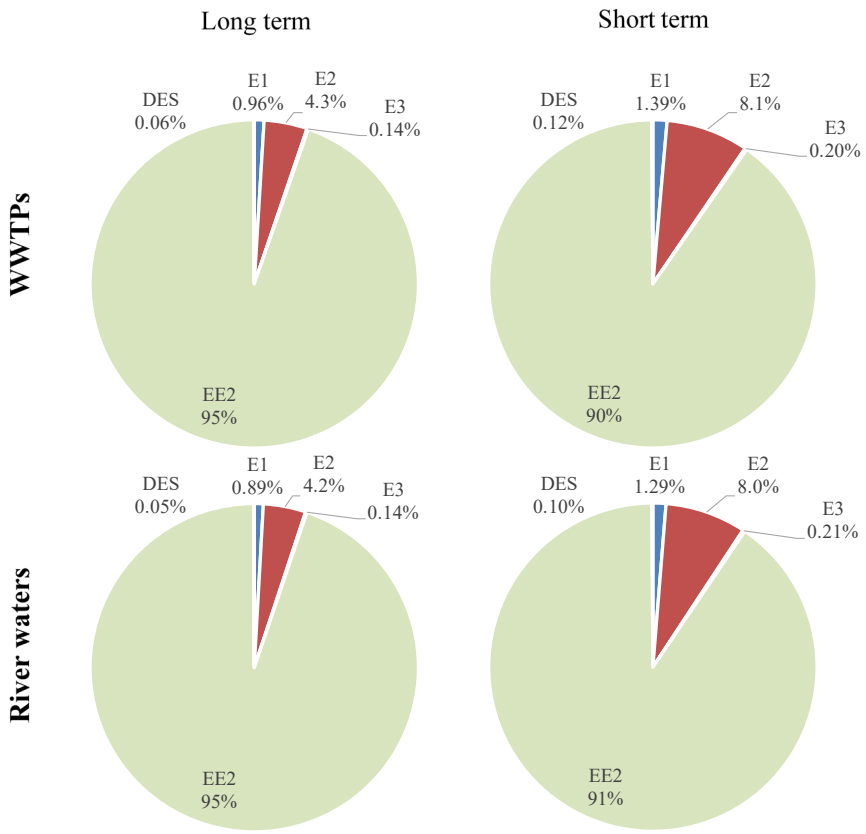

Fig. 6 Composition profile of EEQ of estrogens in WWTP effluents and river waters following long-term and short-term exposures respectively

3.6 Sources of estrogens

Given the potentially high risks to fish induced by local estrogen mixtures, it is important to investigate sources of these estrogens in this region. Human urine is frequently considered to be major sources of both natural and synthetic estrogens in the aquatic environment [4, 32]. A ratio of E3/(E1+E2+E3) > 0.2 in WWTP effluents could be used as an indicator of natural-estrogen release from human excretions [40]. In this study, the average ratio was 0.37 in WWTP effluents with a range of 0.26-0.48 for individual WWTPs in the two seasons. The ratio in the wet season (0.40 – 0.48) was higher compared to the dry season (0.26 – 0.37). Therefore, the natural estrogens reaching WWTPs and ultimately entering rivers are likely to originate mainly from

human excretions.

Taking into account the production of EE2 (approximately 41 kg) for use in contraceptives in China in 2016 with the assumption of a human excretion rate of 100% for EE2 [44, 58] and based on the total human excretion of E1 (5.1 tonnes/year), E2 (1.4 tonnes/year) and E3 (27 tonnes/year) in China in 2010 estimated by a previous study [59], EE2 only accounted for approximately 0.12% of these estrogens excreted by humans in China. The Chinese population increased in 2016, which would probably only cause a slightly higher excretion of natural estrogens. This would not reduce above percentage greatly. This percentage was much lower than that in Netherlands (1%) [32], which indicates a lower prescription rate of pharmaceuticals containing EE2 in China nationally. However, the measured concentrations and percentage of EE2 were comparable to the natural estrogens in the present study region, which indicates a comparable prescription rate of EE2 containing contraceptives in Beijing and Tianjin with that in developed countries such as Netherlands. This aligns with the predicted distribution of EE2 across China by Grill et al. [11] and the conclusion made by Zhu et al. in a previous study [12]. Natural estrogens could be also contained in prescribed pharmaceuticals for human use, however it is difficult to differentiate those naturally excreted and those in prescription form via measurements.

As indicated above, agricultural sources are also potentially important sources of estrogens, since livestock excrete E1, E2 and E3. In most situations effluents or waste from livestock farms will not be treated. The quantity of estrogens present in urine and

faeces of livestock that enter rivers depends on their chemical properties, the distance of livestock farms from rivers and precipitation rates. About 0.61, 8.2, 3.2 and 150 million head/units of live cattle, swine, sheep and poultry respectively were marketed around the years 2016 and 2017 [60-62]. Such production would potentially result in diffuse inputs of estrogens to the study rivers but this remains largely unknown.

As EE2 is only used in human prescriptions and DES is officially restricted in human use, the insignificant correlation of EE2 and DES concentrations for both seasons (Table S9) implies that the restriction of DES use probably has been strictly enforced. Because DES is mostly being used in animals, EE2 and DES could be considered to be indicators of human and livestock sources respectively. E3 significantly correlated to EE2 but not to DES in both seasons, indicating its predominant human sources. All three natural estrogens significantly correlated to EE2 in the wet season, but only two (E2 and E3) significantly correlated to EE2 in the dry season. The correlation coefficient was higher in the wet season than in the dry season. This indicated a closer link of natural estrogens to human sources in the wet season compared to the dry season. This is probably caused by the sewer overflow after precipitation in the wet season, which transported more human excreted estrogens into the rivers. Johnson et al. estimated that 15% of all the estrogens in UK waters were from farm animals, if 1% of steroid estrogens in soils were transported to river waters by overland runoff [63]. Therefore, it will facilitate the contamination control of estrogens, if the proportion of estrogens in river waters derived from livestock excretions can be quantified in this region and across China.

4. Conclusions

Seasonal variation and spatial distribution of concentrations, mass fluxes and composition and the EEQ of five estrogens have been investigated in municipal wastewater and river waters receiving wastewater in the Beijing-Tianjin-Hebei region. With the exception of E1, the other four estrogens in this region showed higher concentrations compared to measurements from other studies. E2, E3 and EE2 were the predominant estrogens in both municipal wastewater and river waters. The high additive EEQ of estrogen mixtures indicated a potentially high risk of adverse effects to fish, both at an individual level and at a population level. EE2 contributed over 90% to the EEQ. As a result, field observations of such effects in the wild fish population is urgently required across China where there is a lack of such data. Although this will require considerable effort, such investigations will ensure a clearer picture of the estrogenic effects to different fish species and ecosystems, along with identifying any potential impact to human health. The impact of the introduction of policy controls on the use of DES has also been addressed in this study. An assessment of the prevalence of DES across the study rivers implies that the animal excretion could be a major source of DES. However, more accurate quantitative estimates are required to assess the sources of different estrogens in river systems in the future, and the effects of human activities need to be explored further. This will ensure that river contamination by estrogens can be controlled and the effects of their presence reduced..

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Appendix A. Supplementary material

Supplementary data to this article can be found online.

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