

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

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1 **Abstract**

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

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

22 1. Introduction

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

38 The Beijing-Tianjin-Hebei region has one of the highest population densities in China.
39 The region also produced approximately 5.3 million tonnes of meat annually in 2016
40 and 2017 [10]. Therefore, large quantities of estrogens (e.g. E1, E2 and E3) could
41 potentially be released from both human and animal excretion, and ultimately enter the
42 Wenyu River, Beiyun River, Haihe River and Yongdingxin River running through
43 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-d₄ (E1-d₄), 17β-estradiol-2,4,16,16,17-d₅ (E2-
77 d₅), estriol-2,4-d₂ (E3-d₂), 17α-ethinylestradiol-2,4,16,16-d₄ (EE2-d₄), were used as
78 internal standards (ISs). All standards and the ISs were purchased from Sigma-Aldrich
79 (UK) with the purity $\geq 97\%$. 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 m³/day) and two major WWTPs
94 in Tianjin (0.15 and 0.4 million m³/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

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

131 **2.4 Quality assurance and quality control**

132 The calibration curve for each target estrogen exhibited a strong linearity ($R^2 > 0.99$)
133 over a wide range of concentrations (several to 500 or 1000 $\mu\text{g/L}$, equivalent to ng/L in
134 water samples) (Table S4). The method quantification limit (MQL) for the selected
135 estrogens ranged 0.47-6.35 ng/L (Table S4). For each batch of samples, reagent blanks,
136 procedural blanks and sample replicates were analyzed to monitor possible
137 contamination and instrumental performance. The experimental procedure was
138 determined to be free of contamination. The relative standard deviations of the triplicate
139 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 ranged 72.6–93.9% 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.

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

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 [\[28\]](#).

161 RPF values varied considerably in the literature especially for EE2, as a result of the
162 selection of different fish test species, endpoints and assay methods. For example, the
163 RPF of EE2 was found to be as high as 30.6 and 40 in inducing vitellogenin (VTG) in
164 adult female zebrafish and adult male fathead minnows, respectively [29, 30], but only
165 5 in inducing intersex of fish in another study [31]. Jobling et al. used different sets of
166 values to estimate EEQ for induction of intersex and VTG, respectively [4]. The range
167 of RPF values for fish could range from 1.19 – 40 for EE2, 0.2 – 0.4 for E1 and 0.024
168 – 0.033 for E3 [4, 32-35]. In this study, RPF values of these estrogens were derived
169 from the relative difference of PNECs (predicted no effect concentrations) protective
170 of reproductive effects in fish following both long-term and short-term exposures [27,
171 34]. PNECs in the case of long-term exposures derived from species sensitivity
172 distributions, and the corresponding RPF values were 0.33, 0.033 and 20 for E1, E3 and
173 EE2; whilst PNECs for short-term exposures derived from no-observed-effect
174 concentrations (NOECs), and the corresponding RPF values were 0.25, 0.025 and 10
175 for E1, E3 and EE2 [34]. RPF values for DES are very limited in the literature, however,
176 0.026, used in this study, was reported based on the median effective dose values [36].

177 **3. Results and discussion**

178 **3.1 Estrogens in wastewater**

179 Although surface runoff from soils contaminated by livestock waste can be an important
180 diffuse input of steroidal estrogens to aquatic environment, WWTP effluents are
181 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 $\mu\text{g/L}$ [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 Σ 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,

204 E2 exhibited the second highest proportion in effluents, however in Tianjin, E3 was the
 205 second most abundant, which, however, has a proportion very close to EE2. Previous
 206 studies have reported EE2 to be present at comparable concentrations to natural
 207 estrogens in WWTP effluents, although annual prescription rates are relatively low (e.g.
 208 50 kg in Germany, production of 41 kg in China) [43, 44]. This could be a result of its
 209 higher stability and the cleavage of the principally excreted glucuronide conjugates [43].
 210 In contrast to this study, E1 has been reported to be the most abundant in WWTP
 211 effluents, mostly due to its poor removal efficiency in WWTPs [40-42, 45]. However,
 212 E3 is the final estrogen human metabolite, and its presence in influents is likely to be
 213 high. E2 is the primary human metabolite. The excretion rates of the two estrogens from
 214 pregnant women is high with the excretion rate for E2 reaching up to 5 mg/day [38].
 215 Therefore, these two estrogens could be more abundant in effluents than other estrogens.
 216 Different effluent compositions from separate studies and locations may be related to
 217 varying human excretion rates, population characteristics including number of pregnant
 218 women, chemical properties and numerous parameters that will affect their removal
 219 efficiencies in WWTPs, including temperature, flow rates and microbial activity [42,
 220 46].

221 **Table 1 Seasonal average concentrations of estrogens in WWTP effluents (mean**
 222 **±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

223 STD: standard deviation

224

225 **3.2 Spatial distribution of estrogens concentration and composition in receiving**
 226 **rivers**

227 The presence of estrogens in river water samples from Beijing to the estuaries entering
 228 Bohai Bay, receiving above municipal wastewater, were analyzed. In all river water
 229 samples, the average total concentration of \sum estrogens was 219 ± 23 ng/L, of which the
 230 three natural estrogens accounted for 62% averagely. The average concentrations were
 231 33, 51, 53, 58 and 24 ng/L for E1, E2, E3, EE2 and DES, respectively. Comparing these
 232 data with other studies worldwide ([Table S6](#)), the concentrations of E1 were comparable
 233 with or lower than other studies; and the concentrations of the other four estrogens were
 234 relatively higher than those from other studies with the exception of data from 139
 235 streams in the USA [\[47\]](#). Lei et al. reported a lower concentration of all five estrogens
 236 in the Yongdingxin River compared to this study [\[48\]](#). Overall, the concentrations
 237 reported in this study were higher than those from many other studies worldwide. This
 238 matches the estimate by previous studies in this region, which have suggested that
 239 emissions were high for four of the estrogens (except DES), even after WWTP removal,
 240 as above mentioned.



241
 242 **Fig. 2. Spatial distribution of average concentrations of Σ estrogens in the two**
 243 **seasons along rivers (A); River names are denoted in italic bold characters; Figure**
 244 **B is an enlargement showing the spatial distribution of the concentration within**
 245 **the dashed-line square in Figure A. The sites named UW1-3 are UW discharge**
 246 **sites; the sites marked with asterisk (*) indicate WWTPs.**

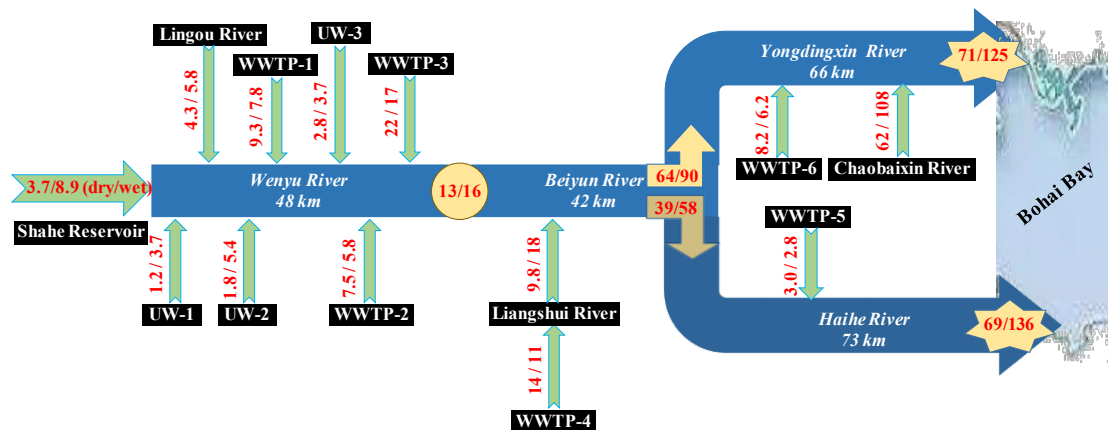
247 The spatial distribution of average concentrations of Σ estrogens in the two seasons
 248 along the study rivers is illustrated in Fig. 2. It has been demonstrated that WWTP
 249 effluents and UW discharges were the major contributors of the estrogens measured in
 250 the study rivers, provided that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

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

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

277 **3.3 Mass fluxes of estrogens along rivers and from WWTP effluents**

278 River flow rates were estimated by measurements of the flow velocity (m/s) and the
279 width and average depth of the river cross section at the sampling site when collecting
280 samples. Average effluent flow rates were obtained from the WWTP managers. The
281 mass fluxes of estrogens were therefore calculated by multiplying measured
282 concentrations and flow rates. The mass flux of Σ estrogens from outlet of the Shahe
283 Reservoir to the Wenyu River was estimated to be 6.3 g/h on average, with a total annual
284 mass loading of ca. 55 kg. The seasonal average mass flux of Σ estrogens from the three
285 UW discharging sites to rivers ranged from 2.4 to 3.6 g/h, with a total mass loading of
286 approximately 81 kg/year, with natural estrogens accounting for ca. 65%. The mass flux
287 of Σ estrogens was the highest from WWTP3 (19 g/h) which is the largest WWTP in
288 Beijing with a 1 million m³/day wastewater treatment capacity ([Table S2](#)). The total
289 input of Σ estrogens from WWTPs 1-3 to the Wenyu River was estimated to be 303
290 kg/year with natural estrogens contributing approximately 62%. The mass flux of
291 Σ estrogens in effluents from WWTP4 was estimated to be 12 g/h, with estimates of 2.9
292 g/h and 7.2 g/h for WWTP5 and WWTP6, respectively. The mass flux of Σ estrogens
293 from one river to another and from tributaries to the mainstream is shown in [Fig. 3](#). The
294 estimated total mass loading of Σ estrogens was approximately 1.8 tonnes/year from the
295 Haihe and Yongdingxin Rivers to the Bohai Bay, with natural estrogens contributing
296 approximately 63.5%.



297

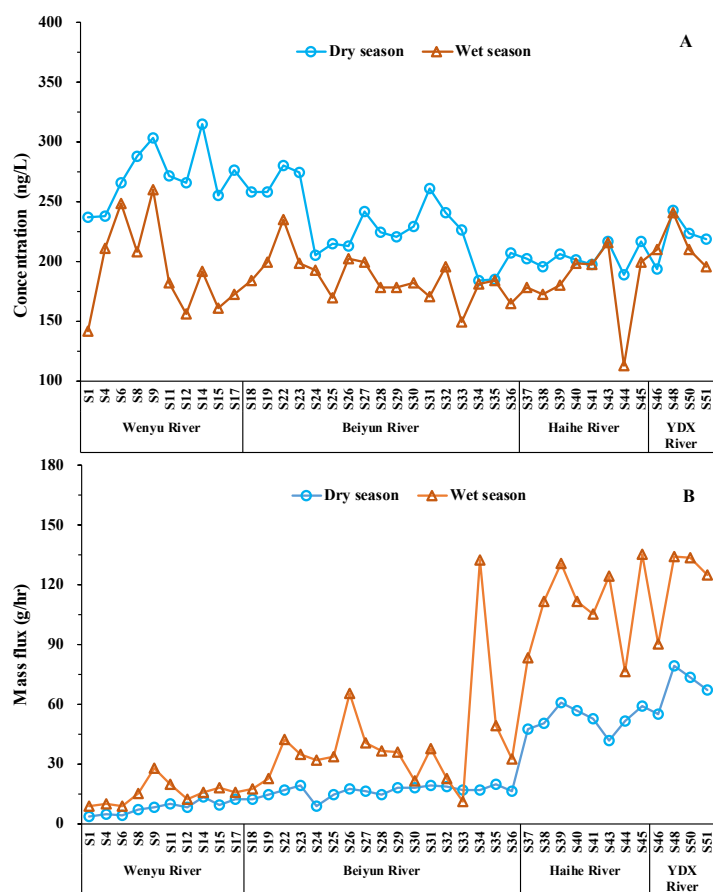
298 **Fig. 3 Mass fluxes of Σ estrogens along rivers and mass loadings from WWTPs to**
 299 **300 rivers (g/h) for both seasons**

300 3.4 Seasonal variation of concentrations and mass fluxes

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

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

322

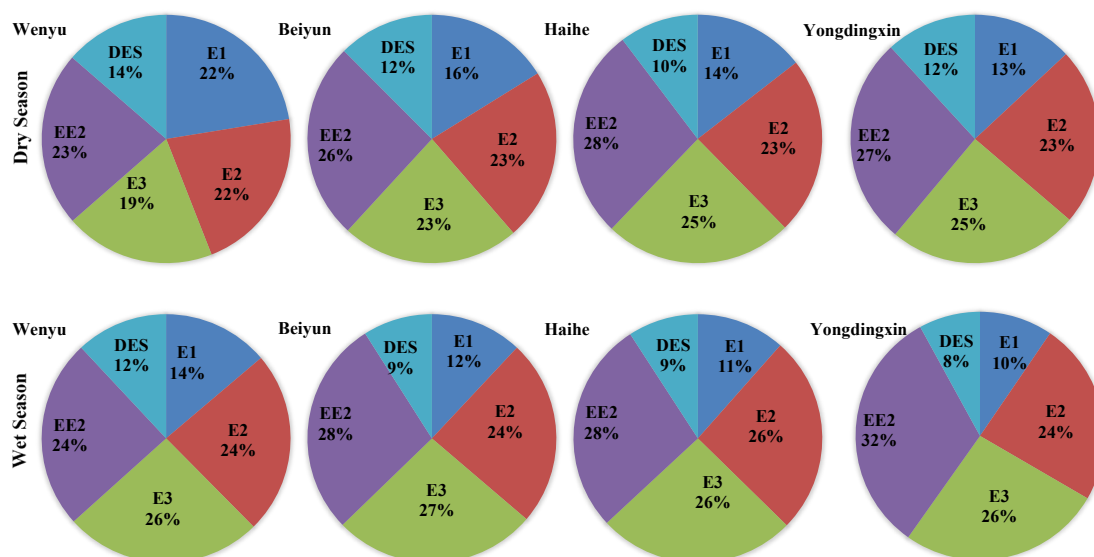


323

324 **Fig. 4 Seasonal concentrations (A) and mass fluxes (B) of Σ estrogens**

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

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



340

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

343 **3.5 Risk assessment of estrogen mixtures**

344 Adverse effects and associated risks were assessed for the mixture of estrogens rather
345 than for individual substances as a result of multicomponent chemical “cocktail” in
346 reality. The EEQ for the five measured estrogens was calculated to provide an
347 assessment of risks to fish induced by the mixture of estrogens. RPF values from both
348 long-term and short-term fish studies were taken from the literature. The corresponding
349 EEQ was compared with the long-term E2 PNEC (2 ng/L) and the short-term E2 PNEC
350 (5 ng/L), respectively [27]. The estimated additive EEQ was $1.2 \pm 0.16 \mu\text{g-E2/L}$ (range,
351 $0.96 - 1.7 \mu\text{g-E2/L}$) following long-term exposures and $0.64 \pm 0.08 \mu\text{g-E2/L}$ (range, 0.5
352 $- 0.86 \mu\text{g-E2/L}$) following short-term exposures in river waters using averaged data.
353 The average additive EEQ was $2.5 \mu\text{g-E2/L}$ and $2.7 \mu\text{g-E2/L}$ in WWTP effluents and
354 UW, respectively, following long-term exposures; and 1.3 and $1.4 \mu\text{g-E2/L}$ in WWTP
355 effluents and UW, respectively, following short-term exposures. The data clearly shows
356 that the EEQ at all sampling sites exceed both the long-term and short-term E2 PNECs,
357 which indicated a high risk to fish from exposure to estrogen mixtures in these rivers.
358 Additionally, the estimated EEQ was higher than the NOECs for inducing intersex (1
359 ng/L) and VTG (5 ng/L) and even higher than the LOECs (the lowest observable effect
360 concentrations) for inducing intersex (10 ng/L) and VTG (25 ng/L) selected by Jobling
361 et al. for their risk assessment [4]. Arlos et al. estimated that an $\text{EEQ} \geq 10 \text{ ng-E2/L}$ was
362 associated with high intersex incidence and severity [54]. Therefore, concentrations of
363 estrogens measured in this study in the target region are likely to have been high enough
364 to cause intersex or VTG etc., which will affect the reproduction and ultimately the
365 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.

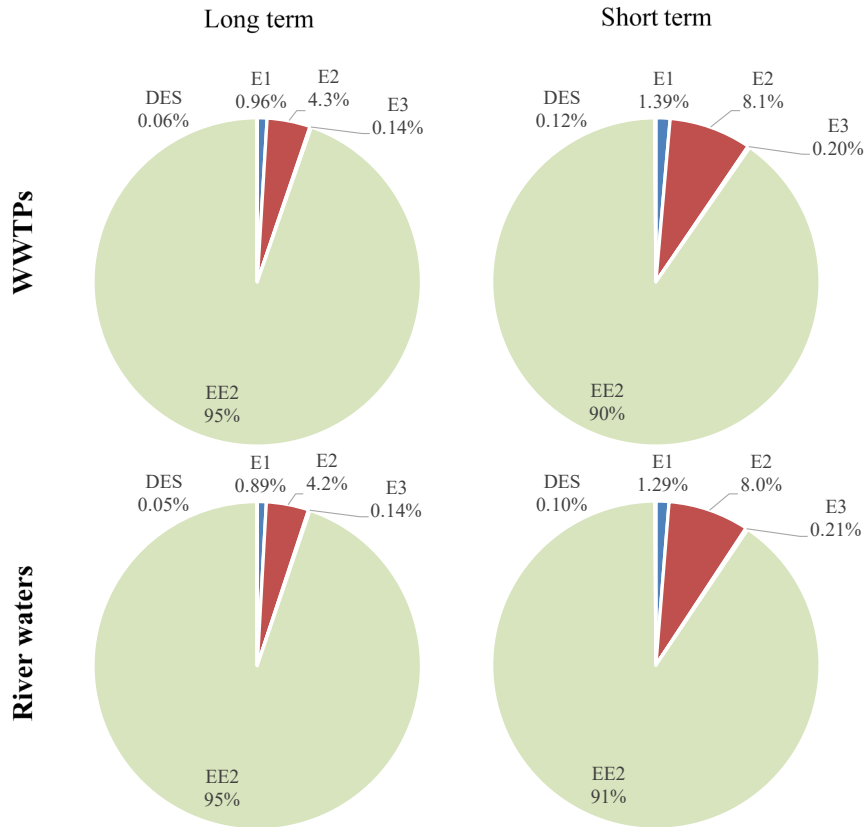


Fig. 6 Composition profile of EEQ of estrogens in WWTP effluents and river waters following long-term and short-term exposures respectively

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3.6 Sources of estrogens

392 Given the potentially high risks to fish induced by local estrogen mixtures, it is
393 important to investigate sources of these estrogens in this region. Human urine is
394 frequently considered to be major sources of both natural and synthetic estrogens in the
395 aquatic environment [4, 32]. A ratio of $E3/(E1+E2+E3) > 0.2$ in WWTP effluents could
396 be used as an indicator of natural-estrogen release from human excretions [40]. In this
397 study, the average ratio was 0.37 in WWTP effluents with a range of 0.26-0.48 for
398 individual WWTPs in the two seasons. The ratio in the wet season (0.40 – 0.48) was
399 higher compared to the dry season (0.26 – 0.37). Therefore, the natural estrogens
400 reaching WWTPs and ultimately entering rivers are likely to originate mainly from
401

402 human excretions.

403 Taking into account the production of EE2 (approximately 41 kg) for use in
404 contraceptives in China in 2016 with the assumption of a human excretion rate of 100%
405 for EE2 [44, 58] and based on the total human excretion of E1 (5.1 tonnes/year), E2
406 (1.4 tonnes/year) and E3 (27 tonnes/year) in China in 2010 estimated by a previous
407 study [59], EE2 only accounted for approximately 0.12% of these estrogens excreted
408 by humans in China. The Chinese population increased in 2016, which would probably
409 only cause a slightly higher excretion of natural estrogens. This would not reduce above
410 percentage greatly. This percentage was much lower than that in Netherlands (1%) [32],
411 which indicates a lower prescription rate of pharmaceuticals containing EE2 in China
412 nationally. However, the measured concentrations and percentage of EE2 were
413 comparable to the natural estrogens in the present study region, which indicates a
414 comparable prescription rate of EE2 containing contraceptives in Beijing and Tianjin
415 with that in developed countries such as Netherlands. This aligns with the predicted
416 distribution of EE2 across China by Grill et al. [11] and the conclusion made by Zhu et
417 al. in a previous study [12]. Natural estrogens could be also contained in prescribed
418 pharmaceuticals for human use, however it is difficult to differentiate those naturally
419 excreted and those in prescription form via measurements.

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

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

428 As EE2 is only used in human prescriptions and DES is officially restricted in human
429 use, the insignificant correlation of EE2 and DES concentrations for both seasons
430 (Table S9) implies that the restriction of DES use probably has been strictly enforced.

431 Because DES is mostly being used in animals, EE2 and DES could be considered to be
432 indicators of human and livestock sources respectively. E3 significantly correlated to
433 EE2 but not to DES in both seasons, indicating its predominant human sources. All
434 three natural estrogens significantly correlated to EE2 in the wet season, but only two
435 (E2 and E3) significantly correlated to EE2 in the dry season. The correlation
436 coefficient was higher in the wet season than in the dry season. This indicated a closer
437 link of natural estrogens to human sources in the wet season compared to the dry season.

438 This is probably caused by the sewer overflow after precipitation in the wet season,
439 which transported more human excreted estrogens into the rivers. Johnson et al.
440 estimated that 15% of all the estrogens in UK waters were from farm animals, if 1% of
441 steroid estrogens in soils were transported to river waters by overland runoff [63].

442 Therefore, it will facilitate the contamination control of estrogens, if the proportion of
443 estrogens in river waters derived from livestock excretions can be quantified in this
444 region and across China.

445 **4. Conclusions**

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

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470 **Appendix A. Supplementary material**

471 Supplementary data to this article can be found online.

472

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