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Chemical and Bioassay Analysis of Estrogen Pollution in the Surface Water of the Tiaoxi River, the Source River for Taihu Lake

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Abstract The estrogen pollution in the Tiaoxi River, which is the main source river for Taihu Lake, was investigated by chemical and bioassay analysis. Most estrogens species, except estrone, were not detected by the chemical analysis using liquid chromatography coupled with tandem mass spectrometry. The concentration of estrone in the samples ranged from ND (below the detection limit) to 17.25 ng/L. The estrogen activity in most water samples was also determined by the yeast estrogen screen. The 17β-estradiol equivalent in the intake of Taihu Lake was 17.60 ng/L and was present in all water samples. This study demonstrates that combining chemical and bioassay analysis is an effective way to detect environmental contamination by estrogen species. Furthermore, the results indicate that the risk of estrogen contamination in the Tiaoxi River should not be ignored.

Keywords Estrogen pollution · LC–MS/MS · YES · Tiaoxi River · Surface water

Endocrine Disrupting Compounds (EDCs) could cause dramatic developmental and reproductive dysfunctions to wildlife and humans. For this reason, environmental contamination and human exposure to EDCs has become a global health concern (Diamanti-Kandarakis et al. 2009). Estrogens are recognized as the most formidable endocrine disrupting compounds in the hormone (Caliman and Gavrilescu 2009). Research has reported that environmental estrogens such as estradiol and estrone are potential risks to the viability of fish and other wildlife (Johnson and Williams 2004). Because waterways are a major sink for many environmental pollutants originating from different sources, numerous natural and anthropogenic estrogens have been detected in water (Shen et al. 2001; Song et al. 2010). Most studies have focused on investigating the estrogenic pollution in water from point source such as wastewater treatment plants (Raman et al. 2004; Ma et al. 2007). Although some studies have focused on the watershed level or diffused pollution of estrogenic pollution, e.g. contamination by estrogens in the Qingdao Licun River and Yangtze River reported by Song et al. (2010) and Zhou et al. (2011), respectively, the non-point source pollution survey has not gained much attention. Therefore, additional investigations of estrogens in surface water from non-point source should be performed.

Modern and powerful analytical techniques, such as gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS), could be used to detect estrogen pollution. However, the chemical analysis is expensive and sometimes identifies only a few types of organic pollutants. Furthermore, it is often difficult to explain possible interactions among compounds and their biological effects through chemical analysis (Shen et al. 2009). In contrast, bioassay analysis could provide a more comprehensive characterization of a sample's potential to modulate a biological pathway, in this case, the estrogen receptor. The combination of chemical and bioassay analysis has been used as an integrated method of detection and has provided comprehensive and accurate

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detection of estrogens in water (Pawlowski et al. 2004; Ma et al. 2007).

The Tiaoxi River, mainly consisting of East, West, South and North of Tiaoxi River, serves as drinking water source and also supplies water for agricultural and industrial purposes for many cities located in northern Zhejiang Province, China. Furthermore, the annual flow of Tiaoxi River is 2.7 billion m^3 and contributes approximately 60 % of the total source water for Taihu Lake, which is the third largest lake in China (Liu et al. 2011). Estrogen contamination of Taihu Lake has been reported and is a source of great concern (Shen et al. 2001; Lu et al. 2011). Meanwhile, the estrogen pollution of the Tiaoxi River may greatly influence the estrogens level of Taihu Lake. However, the estrogen pollution level of the Tiaoxi River has been scarcely reported. Therefore, this study aimed to obtain data to investigate the estrogen pollution in the surface water of the Tiaoxi River through chemical and bioassay analysis.

Materials and Methods

Nine water samples were collected from the Tiaoxi River, 30 cm below the water surface. The sampling sites were described in detail in Fig. 1. All of the samples were placed into brown sampling jars, and 5 % methanol was added to each sample to avoid microbial contamination.

For water sample pretreatment, samples were filtered through 1.0 μm glass fiber filters (APFF) and extracted by hydrophilic-lipophilic balance solid phase extraction columns (6 cc, 500 mg), which had been conditioned with 5 mL of methyl *n*-butyl ether (chemical analysis) or 5 mL of CH_2Cl_2 (bioassay analysis), 5 mL of methanol and 5 mL of deionized water. The columns were eluted three times with 10 mL of methyl *n*-butyl ether/methanol (9:1, v/v) for the chemical analysis or 15 mL of CH_2Cl_2 for the bioassay analysis. The elution was then evaporated to 0.5 mL under a nitrogen stream. Then, the 0.5 mL elution was transferred to a column containing 10 g of Al_2O_3 (50–200 μm, chemical analysis) or 10 g of silica (60–200 μm, 60 A, ultrapure, bioassay analysis). The elution was eluted by 10 mL of acetone/methanol (5:5, v/v; chemical analysis) or 30 mL of methanol (bioassay analysis). The elution was evaporated and conditioned with hexane to 1 mL for the chemical analysis or conditioned with dimethyl sulfoxide (DMSO) to 1 mL for the bioassay analysis (Ma et al. 2007).

The chemical analysis step by liquid chromatography and tandem mass spectrometry (LC–MS/MS) was adapted from an existing method, and the chromatographic gradient was optimized. Briefly, the injected volume was 10 μL, and a gradient with LC-grade methanol/water (70/30 v/v at a flow-rate of 0.2 mL/min) was applied for the separation

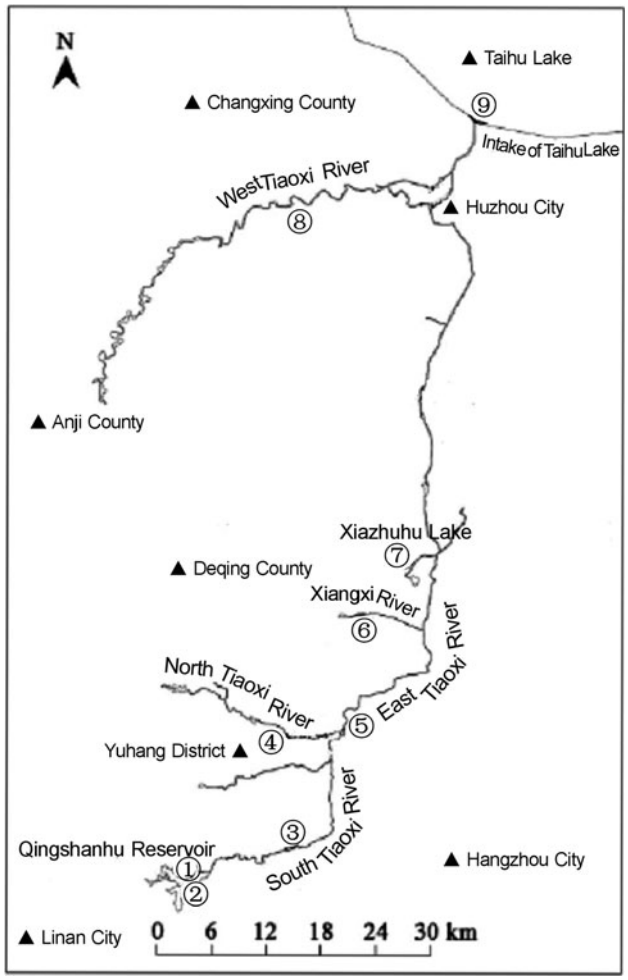

Fig. 1 The study area and sampling sites, ① Qingshanhu reservoir 1# (QSH1), ② Qingshanhu reservoir 2# (QSH2), ③ South Tiaoxi River (STX), ④ North Tiaoxi River (NTX), ⑤ East Tiaoxi River (ETX), ⑥ the Xiangxi River (XX), ⑦ Xiazhuhu Lake (XZH), ⑧ West Tiaoxi River (WTX), ⑨ Intake of Taihu Lake (ITL)

of the four estrogens. A Phenomenex Lura column (1,500 m mm × 2.11 m mm) was used. Ionization was performed with an electrospray source in a negative mode, and the acquisition was achieved in multiple-reaction monitoring (MRM) mode (ion-source temperature, 350°C; ion-spray voltage, −3 kV; collision gas, 30ψ; curtain gas, 10ψ) (Gabet-Giraud et al. 2010).

For yeast estrogen screen (YES) analysis, a volume of 9 mL (50 μM $CuSO_4$-supplemented) of selective medium was mixed with 1 mL of yeast strain. The yeast strain was grown at 37°C, with shaking at 130 rpm for 20 h. The culture solution was diluted with selective medium to an OD_{600nm} of 0.75. Then a volume of 5 μL of 17β-estradiol (E2, positive control), 5 μL of DMSO (negative control) and 5 μL of elution were combined with 0.995 mL of culture solution. Each test culture (200 μL) was transferred into a 96-well plate and incubated at 37°C with 130 rpm

shaking for 2 h. The cell density of the culture was measured at 600 nm. Furthermore, each test culture (50 μL) was transferred to a new 96-well plate, and after the addition of 120 μL of Z-buffer and 20 μL of chloroform, the culture was preincubated for 10 min at 30°C on a shaker. The enzyme reaction was started by adding 40 μL of O-NPG (13.3 mM, dissolved in Z-buffer). The reactions were terminated by the addition of 100 μL of Na_2CO_3 (1 M). After centrifugation, 200 μL supernatant were transferred into a new 96-well plate and the cell density was measured at 420 nm (Ma et al. 2007). Finally, the estrogen activity of water samples was calculated and the estrogen concentration was multiplied by its relative coefficient to obtain the 17β-estradiol equivalent (EEQ) described as Lu et al. (2011).

Results and Discussion

We detected four types of estrogens, however, only estrone was detected by the chemical analysis. The estrogens 17β-estradiol, estriol and diethylstilbestrol were not detected in any of the samples. The average concentration of extracted estrone in Qingshanhu Lake-1, Lake-2, the South Tiaoxi River and Xiangxi River was 8.85, 12.53, 11.69 and 17.25 ng/L, respectively. Estrone was not detected at the other sites (Table 1).

According to the literature, the average concentration of 17α-estradiol, 17β-estradiol and estrone in the dairy feces was 194.6, 104.4, and 262 μg/kg, respectively (Wei et al. 2011). The estrogens level in Taihu Lake ranged from 1.6 to 30.8 ng/L (Shen et al. 2001). The estrogenic activity of industrial wastewaters was found to range from 0.1 to 13.3 ng EEQ/L in Beijing (Ma et al. 2007), and the distribution of estrogens in Qingdao Licun River was 180 ng/L (Zhou et al. 2011). Of even greater concern, the Yangtze River was contaminated by estrogens (Song et al. 2010). The contamination by estrone is evident from our findings, and the risk of exposure to estrogen contamination in the Tiaoxi River should not be ignored.

Table 1 Estrogenic pollution measured by LC–MS/MS (ng/L)

Samples	17β-estradiol	Estriol	Estrone	Diethylstilbestrol
QSH1#	ND	ND	8.85	ND
QSH2#	ND	ND	12.53	ND
STX	ND	ND	11.69	ND
NTX	ND	ND	ND	ND
ETX	ND	ND	ND	ND
XX	ND	ND	17.25	ND
XZH	ND	ND	ND	ND
WTX	ND	ND	ND	ND
ITL	ND	ND	ND	ND

ND not detected

Table 2 Estrogenic activity measured by the bioassay analysis

Samples	Estradiol equivalent (ng/L)	Standard deviation (ng/L)
QSH1#	4.79	1.27
QSH2#	11.42	0.80
STX	3.61	1.37
NTX	1.53	0.49
ETX	1.05	0.11
XX	8.25	6.4
XZH	ND	ND
WTX	5.71	2.07
ITL	17.60	0.67

ND not detected

In each YES assay, a calibration curve was used to calculate the estrogenic activity by the EEQ. The calculated EC_{50} of 17β-estradiol was equal to 7.37 ng/mL. In comparison, the studies in embryonic neurons indicated an EC_{50} of 1–10 ng/mL for 17β-estradiol (Brinton et al. 1997; Brewer et al. 2006). Water samples were evaluated by using the yeast assay (Table 2). Estrogenic activity was detected in all water samples from nine different sites that distributed water to the tributaries and mainstream of Tiaoxi River. Estrogenic activity in Qingshanhu Lake-1, Lake-2, and the Xiangxi River were 4.79, 11.42 and 8.25 ng EEQ/L, respectively. Estrogenic activity measured in samples from the South, the North, the East, and West Tiaoxi River was 3.61, 1.53, 1.05 and 5.71 ng EEQ/L, respectively. There was no estrogen in Xiaduhu Lake, and the estrogenic activity of the intake of Taihu Lake (17.60 ng EEQ/L) was the highest among all the samples.

According to the previous reports, the EEQ of Taihu Lake was estimated in the range of 2.2–12.1 ng/L in 2001 (Shen et al. 2001). Likewise, comparing the E2 induction rates, the EEQ of Taihu Lake was in the range of ND (not detectable) to 10.75 ng/L in 2011 (Lu et al. 2011). It seems that our findings do not match with these reports. These inconsistent results might be due to the different sites of sampling that were used. Meanwhile, our findings show that the estrogen pollution of Taihu Lake may not currently be beyond the environmental regulatory threshold. However, human waste and animal agriculture had been reported to be one major source of estrogen pollution in the United States (Hanselman et al. 2003; Raman et al. 2004). Estrogenic activity in the range of 1.05–17.60 ng/L was detected by the YES analysis in the Tiaoxi River. This level of estrogenic pollution is alarming and should be mitigated immediately.

Table 3 The comparison of the chemical and bioassay analysis (ng/L)

Samples	LC–MS/MS	YES analysis
QSH1#	2.124	4.79
QSH2#	3.007	11.42
STX	2.806	3.61
NTX	ND	1.53
ETX	ND	1.05
XX	4.140	8.25
XZH	ND	ND
WTX	ND	5.71
ITL	ND	17.60

ND not detected

For comparison of chemical and bioassay analysis, the results of LC–MS/MS were multiplied by the relative coefficient (0.25) to obtain the EEQ and compared with the results of the YES analysis (Table 3). The EEQ from the chemical analysis ranged from ND to 4.14 ng/L, and the EEQ from the bioassay analysis ranged from ND to 17.60 ng/L. The EEQ of the bioassay analysis was apparently higher than the EEQ results of the chemical analysis. Because of the narrow detection limit of the chemical method, only a limited number of estrogens could be detected. There may be variety of estrogen substances in water, and the bioassay analysis can reveal comprehensive effects of estrogen, thus explaining why the values of the YES analysis were higher than those of the chemical analysis (Ma et al. 2007).

The Pearson correlation coefficient was calculated to determine correlations between the YES analysis and LC–MS/MS. No significant linear correlation between these two sets of data was evident. However, the YES analysis is a rapid, efficient, low-cost method that uses coarse filters for a large number of samples. Hence, the method could be considered a supplement to the LC–MS/MS and may provide more comprehensive and accurate detection of estrogen substances in water.

In summary, the combination of chemical and bioassay analysis, as an integrated method of detection, proved to be important for comprehensive and accurate detection of estrogen substances in waterways. Furthermore, estrogenic activity was detected in most of the water samples of the Tiaoxi River, which indicates that the risk exposure to estrogen contamination in the Tiaoxi River should not be ignored.

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