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Assessment of *in vivo* estrogenic response and the identification of environmental estrogens in the Yangtze River (Nanjing section)

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ABSTRACT

Environmental estrogens in the aquatic environment have been shown to be responsible for the feminization of fish. The estrogenic content of the Yangtze River (Nanjing section – referred to as the studied area herein) was assessed using a combination of bioassay and chemical analysis. The *in vivo* bioassay was conducted by exposing adult male goldfish (*Carassius auratus*) to different concentrations of river water (25%, 50% and 100%) sampled from three representative sections of the studied area. Chemical analysis of estrogens in water from the three representative sections was conducted using solid phase extraction-gas chromatograph (SPE-GC) detection. The assay showed significant serum vitellogenin (VTG) and 17β-estradiol (E_2) induction and gonad atrophy in the treated fish. The strength of *in vivo* estrogenic responses in the three representative sections is in the order of Jiangxinzhou section > Daqiao section > Sanchahe section. The result is consistent with the levels of water estrogens determined from the chemical analysis. Steroidal estrogens were the major causal agents responsible for the estrogenic responses in the Jiangxinzhou and Daqiao sections, while phenolic estrogens were the main contributors in the Sanchahe section. The results of these *in vivo* bioassay and chemical analysis demonstrate that fish in the Yangtze River are exposed to environmental estrogens and are at a risk of feminization.

1. Introduction

Recently, feminization of fish due to long term exposure to low concentrations of estrogens in aquatic environments have caused great concern throughout the world. Generally, environmental estrogens have been detected at ng L^{-1} levels in the waters studied (Liu et al., 2004; Hibberd et al., 2009). But, even at these levels they were found to be biologically active (Brion et al., 2004; Pawlowski et al., 2004). Estrogen-induced feminization presents itself in a number of different ways: a sex ratio biased in favor of females; occurrence of intersex individuals (having simultaneous presence of ovarian and testicular tissue in the same gonad); delayed sexual maturation; reduction in gonadosomatic index (GSI); histological alterations in the gonads; disruption of spermatogenesis; decrease in fertilization rate; alterations in circulating steroid concentrations; altered biomolecules; alterations in reproductive behavior; etc. (Milnes et al., 2006). Simultaneous development of male and female tissue in the gonads and testicular atrophy were observed in wild carp (*Cyprinus carpio*) sampled in a section of the Anoia River (NE Spain) (Solé et al., 2003). Higher levels of plasma vitellogenin (VTG) were found in male brown trout (*Salmo trutta*) sampled from six Danish streams impacted by sewage effluent, compared to males from unaffected reference sites (Bjerregaard et al., 2006). Male wild goldfish (*Carassius. auratus*) sampled in the Young-San River in Korea, showed high concentrations of VTG and lowered GSI (Li et al., 2009). Feminization of fish have also been reported in China, the Netherlands, South Africa, and the Czech Republic (Hu et al., 2003; Vethaak et al., 2005; Wepener et al., 2005; Randak et al., 2009). Feminized males are most often observed near wastewater outfalls or in areas receiving large amount of domestic or industrial wastewater.

Discovering trace estrogens in natural waters requires sensitive and robust test tools and analytical methods. Several chemical analyses (e.g. LC, GC, GC–MS) are highly sensitive for the (xeno) estrogens and can identify and quantify trace levels in water samples. However, chemical analyses take no account of the additive, synergistic or antagonistic effects of structurally different xenoestrogens found in water. In contrast, bioassays can. They reflect the integrated responses of living organisms to all the estrogenic chemicals present in a system, including those that might be

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missed by chemical analyses. They allow the identification of biomarkers for adverse effects of pollution on an indicator fish, another useful tool. The assessment of estrogenic pollution in water should therefore include not only chemical analyses of pre-selected compounds, but also biological assays and tools as well. A combination of biological tools and chemical analyses has become a valid method for evaluating the estrogenic effects of water samples and identifying the causative agents.

The Yangtze River is the largest river in China. Nanjing is a highly urbanized city in the Yangtze River Delta, which is the most developed area in China. In Nanjing, a great deal of effluent from wastewater treatment plants (WWTPs), untreated sewage and upstream wastewater enters the Yangtze River and many pollution zones have formed along the river. In recent years, the number of fish has begun to decrease and fish have tended to be smaller and younger. Fish are often sickly and the number of deformed individuals has increased (Peng, 2006). The objective of this study is to investigate estrogenic effects and identify the main causative agents in the water samples taken from three representative sections of the studied area using an *in vivo* bioassay and a solid phase extraction-gas chromatograph (SPE-GC) analysis. Serum VTG, 17β-estradiol (E_2) and GSI were used as biomarkers in the *in vivo* bioassay. Frequently reported potential causative substances including steroidal estrogens (estrone (E_1), E_2 and estriol (E_3)), synthetic estrogens (17α-ethynylestradiol (EE_2) and diethylstilbestrol (DES)) and phenolic estrogens (4-*tert*-octylphenol (4-*t*-OP), nonylphenol (NP) and bisphenol A (BPA)) were selected as the target estrogens in the SPE-GC analysis.

2. Materials and methods

2.1. Reagents

C18 solid phase extraction cartridges (5 g, 20 mL volume) were purchased from Beijing Zhenxiang Industrial Trade Co., Ltd. (Beijing, China). Purified goldfish vitellogenin and primary antibody (rabbit anti-goldfish vitellogenin) were obtained from the College of Marine Life Sciences, Ocean University of China (Qingdao, China). (Phenylmethyl) sulfonyl fluoride (PMSF), heparin sodium and Tween-20 (with purities >99%) were purchased from Nanjing Sunshine Biotechnology Co., Ltd. (Nanjing, China). Phosphate-buffered saline, non-fat milk, and goat anti-rabbit IgG labeled with alkaline phosphatase (AP) were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). p-Nitrophenylphosphate (pNPP) was purchased from Shanghai Boyun Biotech Co., Ltd. (Shanghai, China). Coomassie brilliant blue G-250 (Ultra Pure Grade) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Bovine serum albumin (with purity >98%) was purchased from Shanghai Huixing Biochemistry Reagent Co., Ltd. (Shanghai, China). E_2 assay kit was purchased from Adlitteram Diagnostic Laboratories Inc. (USA). Standards of OP, BPA, E_1, E_2, EE_2 were purchased from Sigma Chemical Company (St. Louis, MO, USA). DES, NP, E_3 were purchased from Labor Dr. Ehrenstorfer (Augsburg, Germany). The derivatization agent *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% of trimethylchlorosilane (TMCS) was purchased from Regis Corporation (Massachusetts, USA). Pyridine (HPLC grade) and methanol (HPLC grade) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other chemicals were of analytical grade and were obtained from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China).

A stock solution of the individual standards was prepared in methanol at a concentration of 1 g L^{-1} and stored in a glass vial at −20 °C. The working solutions of the individual standards and their mixtures were prepared by serial dilution of stock solutions with methanol and stored at 4 °C.

2.2. Water sampling

Based on the hydrology of the studied area and the effluent discharge points from WWTPs in Nanjing, three representative river sections were assigned near to the main inlets of the Yangtze River and principle WWTP outlets. They were the Jiangxinzhou section, Sanchahe section and Daqiao section. The location of the three representative sections is shown in Fig. 1. Water samples were collected in April 2009. All of the sampling equipment was disinfected with a weak bleach solution, after which it was rinsed with tap water and then distilled water. In order to prevent bacterial growth, methanol (5‰) was added to each water sample immediately after collection.

2.3. Test animals

Adult male goldfish (*C. auratus*) weighing 33.8 ± 2.1 g were obtained from Nanjing Fuzimiao Flower and Bird Market. The fish were acclimatized for 2 weeks in dechlorinated municipal water prior to experimentation, during which time they were fed with commercial fish food (Nanjing, China) once a day. Feces and uneaten food were removed every other day by suction. A 50% water change was performed every other day. Water temperatures ranged from 16 to 18 °C. Fish were not fed for 24 h prior to the experiments and no food was provided during the test period.

2.4. In vivo bioassay

In order to investigate effective concentration of environmental estrogens in the three representative sections of the studied area, experimental solutions were prepared by serial dilution of the river water (25%, 50% and 100%) in dechlorinated municipal water. Randomly assigned fish were kept in 30-L glass tanks (40 × 25 × 30 cm) containing 20 L of various experimental solutions under constant aeration. A semi-static test was conducted by replacing

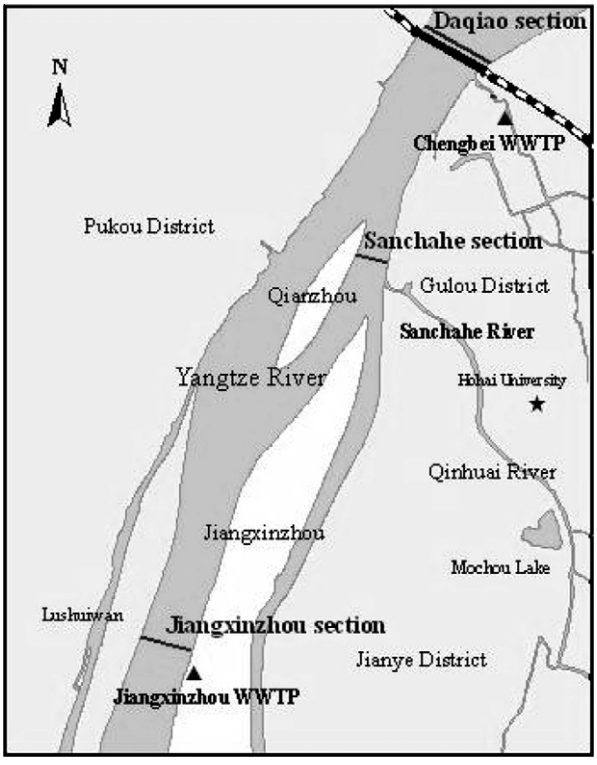

Fig. 1. Location of the three representative sections.