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# Effects and bioaccumulation of 17β-estradiol and 17α-ethynylestradiol following long-term exposure in crucian carp

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ABSTRACT

Bioaccumulation and effects of 17β-estradiol (E2) and 17α-ethynylestradiol (EE2) were assessed by crucian carp (*Carassius auratus*) following single and binary mixture exposures in flow-through exposure system for 16 months. In comparison with water control (DWC) and solvent control (SC), a significant reduction in body weight, body length and gonadosomatic index (GSI), and increase in hepatosomatic index (HSI) and plasma vitellogenin (VTG) levels were observed, in a time- and concentration-dependent manner. Bioconcentration factors (BCFs) of E2 and EE2 in fish muscle ranged from 3.2 to 40 and from 64 to 123, respectively. Crucian carp were found to be more sensitive to EE2 than E2. The bioaccumulation and toxicological effects in binary mixture exposed fish (mixture of E2 and EE2) were more significant than exposure of individual compound. Crucian carp is sensitive to E2 and EE2 in long-term laboratory exposure experiments and can be used as a potential model species for investigating the toxicity of hormones.



## 1. Introduction

The detection of populations of intersex fish in freshwater and estuarine environments of many countries has been associated with exposure to estrogenic chemicals in wastewater effluents (Gibson et al., 2005). Toxicity identification and evaluation studies, directed at isolating and identifying the major estrogenic chemicals present in wastewater treatment plant (WWTP) effluents, have shown that the most active estrogenic fraction ( $>$ 80% total activity in domestic effluent) contains steroid estrogens, such as the natural estrone (E1), 17β-estradiol (E2) and estriol (E3), as well as the synthetic 17α-ethynylestradiol (EE2) (Desbrow et al., 1998; Rodgers-Gray et al., 2001; Tyler et al., 2005). In the last two decades, many studies have reported the occurrence and fate of steroid estrogens in WWTP effluents and receiving waters around the world (Liu et al., 2012; Braga et al., 2005; De Mes et al., 2005). Although those compounds have been found in aquatic environments at ng/L levels, laboratory studies have demonstrated that they are sufficient to induce at least some of the feminizing effects in caged and wild fish (Tyler et al., 2005; Zhang et al., 2011).

To date, most investigations about the uptake and accumulation of steroid estrogens focus on gonochorism fish species, such as brown trout (*Salmo trutta fario*) (Knudsen et al., 2011; Schubert et al., 2008), rainbow trout (*Oncorhynchus mykiss*) (Gibson et al., 2005; Thorpe et al., 2003; Tyler et al., 2005), fathead minnow (*Pimephales promelas*) (Filby et al., 2010; Panter et al., 1998; Panter et al., 2000; Lavelle and Sorensen, 2011), pipefish (*Syngnathus abaster*) (Sárria et al., 2011), and gray mullet (*Mugil cephalus*) (Aoki et al., 2011), limited studies are about gynogenesis species. In this study, a natural gynogenesis species crucian carp (*Carassius auratus*) (also called high-back crucian carp in China) was chosen as the test species, which is a native and important economic fish species in Dianchi Lake (the largest plateau lake in China) (Xiao et al., 2011; Zan, 1982; Zhou et al., 2000). Crucian carp can maintain the stability of species and has a remarkably significance for the local ecological effect to study the adverse effects of endocrine disrupting chemicals (EDCs) on it. Our previous researches have showed that steroid estrogens were detected in crucian carp collected from wild and net-caged in Dianchi Lake, as well as effluent-exposed in waste water treatment plant (WWTP) (Huang et al., 2013; Liu et al., 2011b, 2012; Wang et al., 2013). These results suggested that crucian carp were sensitive to steroid estrogens and could be used as a new potential model species for investigating the toxicology of steroid estrogens, however, further research is needed.

Dianchi Lake located in the southwest of China and adjacent to Kunming City, is the primary reserve source of domestic water for Kunming's 6.8 million residents and supplies water for the growing industrial and agricultural sectors of the city. With the rapid economic growth and urban development, Dianchi Lake and its 21 main inflow rivers have been seriously polluted by wastewaters from industry, agriculture and daily life. In recent years, the

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pollution of EDCs in Dianchi Lake, especially steroid estrogens, has raised public concern. Steroids were detected in all surface water and sediment samples collected from ten sites of the lake. The concentrations of E1, E2, E3 and EE2 in water were ranging between 1.2 and 22.7 ng/L, not detected (nd)-8.3 ng/L, nd-5.3 ng/L and nd-4.4 ng/L, respectively. These steroid estrogens may have the adverse biological effects toward aquatic organism (Huang et al., 2013). Due to the continuous input of untreated wastewaters and effluent from eight WWTPs of Kunming, high bioaccumulations of steroid estrogens were found in muscle, gill and liver of wild fish species (crucian carp, carp and silvery minnow) collected from four sites of Dianchi Lake (Huang et al., 2013; Liu et al., 2011b).

Compared with wild fish species and fish controls caged in Dianchi Lake, a significant reduction in gonadosomatic index (GSI) and increase in hepatosomatic index (HSI) and plasma vitellogenin (VTG) levels were observed in effluent-exposed crucian carp (Liu et al., 2012; Wang et al., 2013). It has pointed out that steroid estrogen bioconcentrate in crucian carp muscle may account for the biological effects associated with exposures to WWTP effluents, however the impact mechanism was not clear. Most toxicity data available are restricted to acute toxicity test or short-term exposure experiment in laboratory. Therefore, the lack of long-term exposure data often limits their application in the assessment of biological effects and environmental risk of EDCs in aquatic environment. So laboratory long-term exposure of crucian carp with concentration control of pollutant needs to be investigated.

The main objective of this study was to assess the biological effects toward crucian carp of long-term exposure to E2 and EE2 in laboratory flow-through exposure system for 16 months. It is important to clarify not only the hazard posed by each chemical independently, but also recognize the interactions created under mixture exposure. Then ascertain whether crucian carp can be used as a potential model species to investigate the toxicology of EDCs. To achieve this, five increasing concentrations of E2 (1, 3.2, 10, 32 and 100 ng/L), EE2 (10, 32, 100, 320 and 1000 ng/L) and two combination concentrations of E2 and EE2 (32 ng/L E2+3.2 ng/L EE2 and 100 ng/L E2+10 ng/L EE2) were applied. We measured mortality rate, growth, GSI, HIS, VTG and bioconcentration factor (BCF) as endpoints. To our knowledge, this is the first report about laboratory long-term exposure to E2 and EE2 in crucian carp.

## 2. Materials and methods

### 2.1. Test chemicals

The test chemicals, E2 and EE2 (purity $>97\%$), internal standard (5*a*-androstane) and surrogates (estrone-$d_4$) were purchased from Sigma-Aldrich (USA). Derivatization reagent, N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were purchased from Fluka (Germany). To prepare stock solutions, individual compounds were initially dissolved in 100% HPLC-grade methanol (Fisher Scientific), sealed and stored at 4 °C.

Organic solvents (methanol, dichloromethane, ethyl acetate, hexane and cyclohexane) used for sample processing and analysis were HPLC grade. Methanol, ethyl acetate and cyclohexane were purchased from Merck (Germany). Dichloromethane and hexane were supplied by J. T. Baker (USA). Solid Phase Extraction (SPE) cartridge of Sep-Pak C18 (6 mL, 500 mg) was obtained from Waters (Milford, MA, USA). All glassware was cleaned by SC 1160 automatic bottle washer (Salvis Lab, Switzerland) and then pyrolysed at 450 °C for 4 h prior to use.

### 2.2. Experimental fish

The freshwater crucian carp (*C. auratus*) (Teleostei: Cyprinidae) was used as the test fish and it was suitable for the long-term toxicity testing as demonstrated in wastewater treatment plant effluents exposure study (Liu et al., 2012; Wang et al., 2013). In this study, juvenile crucian carp were obtained ($4.23 \pm 0.7$ g each) from breeding stocks at Fisheries Research Institute of Yunnan Province, China. A total of 1500 juvenile crucian carps were maintained in the laboratory supplied continuously with dechlorinated tap water for 2 weeks prior to the exposure experiment. All the juveniles used were expected to be females due to gynogenic characteristics of the species. At the beginning of the experiment, 100 fish were randomly allocated to each of fourteen stainless steel tanks (0.7 m (length) $\times$ 1.2 m (wide) $\times$ 0.6 m (depth)) with a working volume of 450 L. The control parameters of water quality included pH (6.9–7.9), oxygen concentration (5.0–7.0 mg/L) and temperature (20.0–25.0 °C). Fishes were subjected to a photoperiod of 16:8 h (light: dark) and generally fed with a commercial granular food at a rate of 0.1% body weight once per day. To avoid the build-up of excessive bacterial colonies, surplus food and feces were removed daily.

### 2.3. Ethical statement and approval

This study and the consent procedure were approved by the local ethical review committee at the Fisheries Research Institute of Yunnan Province and the Kunming University of Science and Technology.

### 2.4. Test apparatus

A continuous flow-through exposure system was employed by Pan et al. (2010) (Fig. 1) (ZL 201020130648.8). Each stock solution was delivered to a mixing vessel at a flow rate of 1 μL/min using the multi-syringes pump (LSP10-1B, Longer, USA) equipped with ten syringes of 10 mL. The syringes were filled every week with stock solutions containing either E2, EE2, mixture of E2 and EE2 or solvent (methanol) alone. The stock solution was dilution by the incoming dechlorinated tap water delivered to a mixing vessel by a peristaltic pump at a flow rate of 300 mL/min. The working solution, at the desired nominal concentration, was then delivered to the appropriate test tank by gravitation. Another water control tank received dechlorinated tap water only at the same flow rate. The solvent control tank also received the same addition of methanol, such that the final concentration of methanol in all treatment tanks was 0.002%. The flow rates of dilution water and stock solutions were checked at least once daily. The flow rate was relatively high, providing a replacement every 24 h, which was intended to minimize the probability of biotic or abiotic degradation or loss of the test substances. The materials of water pipes and chemical tubes were used glass or teflon. The tanks and fish shelters were made from stainless steel.

### 2.5. Experimental design

In acute toxicity test, the 96 h half-lethal concentrations (LC50) of E2 and EE2 to juvenile crucian carp was 0.403 mg/L and 0.149 mg/L, respectively. Different laboratory exposure groups were set up based on the LC50 and the concentration of E2 and EE2 in real wild water environment. These concentrations for the mixture treatments were selected on the basis of middle value of the individual experiment. Crucian carp were exposed to E2 at concentrations of 10, 32, 100, 320 and 1000 ng/L, EE2 at concentrations of 1, 3.2, 10, 32 and 100 ng/L, and binary mixtures of E2 and EE2 at concentrations of 32+3.2 ng/L and 100+10 ng/L.