Environmental Science & Technology

Article

pubs.acs.org/est

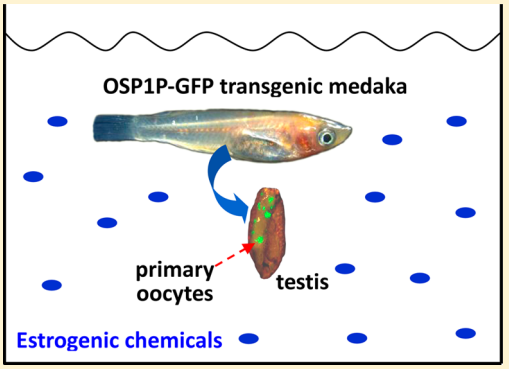
Biosensor Medaka for Monitoring Intersex Caused by Estrogenic Chemicals

Yanbin Zhao, Chen Wang, Shuang Xia, Jieqiong Jiang, Rui Hu, Guanxiang Yuan, and Jianying Hu*

MOE Laboratory for Earth Surface Processes, College of Urban and Environmental Sciences, Peking University, Beijing 100871, China

Supporting Information

ABSTRACT: Estrogenic chemicals can induce intersex in fish species leading to disturbance of spermatogenesis and impairment of reproductive success. To overcome the shortcomings of conventional histopathological observation on intersex (low sensitivity, relatively poor accuracy, long experimental periods, as well as laborious and time-consuming), we generated a pMOSP1-EGFP transgenic medaka fish model. In this transgenic fish, the green fluorescence protein (GFP) reporter gene was derived by the regulatory elements of the OSP1 gene, which is a specific and sensitive molecular biomarker for indicating intersex occurrence in male medaka fish exposed to estrogenic chemicals. The transgenic GFP was faithfully expressed in ovaries and in testes with intersex, perfectly mimicking the expression pattern of endogenous OSP1. In intersex testis, the diameters of primary oocytes which could be distinguished by GFP fluorescence observation were as small as 10 μm, lower than that (more than 20 μm) which is observable by histopathology. Using the novel transgenic medaka fish, intersex was observed after 90-day exposure to 0.75 ng/L 17α-ethinyloestradiol (EE_2) (0−90 dph), but only at concentrations of at least 1.38 ng/L EE_2 by histopathology. An effectiveness of a short-term in vivo assay for screening estrogenic chemicals that can monitor intersex appearance at early sex developmental stage (about 30 dph) in male medaka fish was also demonstrated by assessing the intersex induction of EE_2, 17β-estradiol and 4-nonylphenol. This newly developed assay provides an enhanced ability for screening and testing estrogenic chemicals with the potential to induce intersex and studying their biological impacts.



INTRODUCTION

Natural and synthetic estrogenic compounds derived from industrial wastes, pesticides, and sewage, pollute the aquatic environment and cause endocrine and reproductive impairments in wildlife and humans.[1−5] Exposure to environmental estrogens alters reproductive physiology and morphology, especially in freshwater and marine fish species, resulting in gonadal growth retardation, testicular degeneration, female-specific protein induction, and intersex occurrence in males.[2,3,6] To avoid the potential human and environmental health risk associated with exposure to estrogenic chemicals, international organizations and countries have validated battery screening and testing assays of estrogenic chemicals for mammals, amphibians, and fish.[7,8]

Several in vitro and in vivo screening and testing assays for estrogenic chemicals are currently available. In vitro assays are usually based on specific mechanisms of action, for example, estrogen receptor (ER)-ligand binding assays and ER-activated cell lines. These assays are rapid and simple, but are difficult to extrapolate to complex signaling and regulation of intact endocrine systems in vivo[9] and are less valid than in vivo assays due to their uncertainties and limited understanding of the differences in xenobiotic metabolism, bioavailability, and toxicokinetics.[10,11] Currently, several important in vivo screening assays for estrogenic chemicals have been developed based on observation end points such as morphological observations, vitellogenin induction, and gonadal histology. Especially, recently published assays in zebrafish which are based on the estrogen receptor (ER) or marker genes involved in gonadal differentiation and development regulated transgenic GFP expressions exhibit application perspectives for assessing estrogenic activity of a chemical due to their sensitive, fast, and convenient advantages.[12−16] In these in vivo assays, intersex is a specific end point related to disturbance of spermatogenesis and impairment of reproductive success caused by estrogenic chemicals, such as the reduction of milt volume, sperm density, and fertility,[17−19] and its incidence has been considered as a direct evidence of feminization of wild fish exposed to estrogenic chemicals.[2,20,21] While conventional histopathological observation on intersex has been well established as described in the OECD Histopathology Guidance Document,[22] intersex can be easily overlooked due to limited observation area of the testes, which may also be

Received: June 18, 2013
Revised: January 20, 2014
Accepted: January 22, 2014
Published: January 22, 2014

   dx.doi.org/10.1021/es4052796 | Environ. Sci. Technol. 2014, 48, 2413−2420

deformed, atrophied, or lost, and the skilled labor, special techniques, equipment, and chemicals required for the procedure, leading to excessive costs. In addition, the diameters of observable primary oocytes using histopathological observation usually need to be greater than 20 μm in fish,[20,23] and therefore oogonia and early primary oocytes cannot be distinguished from spermatozoids and other somatic cells with diameters of approximately 5−10 μm.[24] Further, it is well accepted that fish in the early developmental stage tend to be more sensitive to intersex induction than at the adult stage,[22,26] and therefore exposure usually starts from just hatching and continues to sexual maturity for histopathological observation. Such assays require a relatively long time period, usually 3−6 months for medaka.[27,28] Thus, to overcome the shortcomings of conventional histopathological observation and enhance estrogen chemical screening capability, there is a need to develop an assay capable of more quickly and accurately detecting intersex with high sensitivity.

Japanese medaka (*Oryzias latipes*), currently included in OECD chronic test guidelines, are one of the most popular model fish species used for endocrine disruption studies[28−30] due to their definite sex specific markers (chromatophores and sex determination locus) and secondary sex characters (shape of the anal and dorsal fin).[30] Transgenic medaka, with considerable potentials, have been widely used for screening and testing EDCs,[31,32] and have greatly enhanced the understanding on their mechanisms of effects and the assessment on their potential ecological impacts. Our previous paper has proved that OSP1 gene is a sensitive molecular biomarker to indicate intersex occurrence in male medaka fish exposed to estrogenic chemicals.[24] Based on the Ensembl and NCBI database, this OSP1 gene has six additional copies of OSP cDNAs and identified only in the medaka genome. These proteins contain signal peptides and may form a specific family of secreted proteins in medaka fish which would play important roles in female oogenesis. In the present study, we established an intersex-specific biomarker-responsive transgenic medaka strain to assess the estrogenic activity of chemicals. This in vivo visual reporter system harbored the green fluorescence protein (GFP) gene which was driven by the promoter region of medaka endogenous OSP1. We demonstrated much higher sensitivity for evaluating the incidence and severity index of intersex using this visual reporter system compared with histopathological approaches, and we further established a short-term in vivo assay for screening estrogenic chemicals since this system can monitor intersex appearance at early sex developmental stages of male medaka fish.

MATERIALS AND METHODS

Fish. Japanese medaka, orange red strain, were cultured in flow-through tanks under conditions that facilitated breeding (16:8 light/dark cycle) and were fed live brine shrimp (*Artemia nauplii*) twice daily. Water used in the experiment was filtered through activated carbon and had a hardness of 8.1 ± 0.1 mg/L $CaCO_3$, pH 7.7 ± 0.2, dissolved oxygen 7.8 ± 0.3 mg/L, and temperature 25 ± 1 °C.

Generation of pMOSP1-EGFP transgenic fish. Two specific primers (5′-CGGGG TACCTT-CAACTTGTGTGGGGTAGATGT-3′ and 5′-TCCCCCCGGGTCTGCTGGTTGCT ATTGACTGAT-3′) for the flanking regions of the OSP1 gene were designed based on medaka genome information (http://www.ensembl.org/Oryzias_latipes/Info/Index, transcript ID of OSP1: ENSORLT00000017571). A 5′ flanking region 2067 bp fragment of the proximal promoter region was amplified by PCR, sequenced completely, and then inserted into *Kpn*I/*Xma*I restriction enzyme sites of the pEGFP-1 vector (BD Biosciences Clontech) to construct the chimeric plasmid, pMOSP1-EGFP. The main sequence of pMOSP1-EGFP plasmid is shown in Figure S1, Supporting Information. The pMOSP1-EGFP plasmid was then linearized with the restriction enzyme Sac I and microinjected into fertilized eggs of d-rR strain before the first cleavage at a final concentration of 100 ng/μL. Injected embryos were incubated at 26 °C in embryo-rearing medium and reared to adulthood. Sexually mature founder fish were individually mated with wild-type fish. The PCR with primers (5′-TAGTCTTGGGCATCAGTCAA-TAGCA-3′ and 5′-CAACACTCAACCCTATCTCGGTC TATT-3′) was adopted to examine the existence of the GFP transgene from pooled embryos. Of the 151 founders screened, three germ-line transmitted transgenic founders were identified. These transgenic F0 individuals were mated with nontransgenic d-rR to establish F4 homozygous transgenic fish. One strain, which showed a relatively strong fluorescence compared to the others, was selected for subsequent analyses.

Exposure Design. For comparison with histopathological observation, medaka larvae collected on the first day after hatching were used in each of the 0.5, 1, 2, 4, 8, 16, and 32 ng/L EE_2 exposure groups and the vehicle control (0.005% DMSO) (two tanks per treatment). The EE_2 concentrations used were designed to induce different intersex severity indexes based on precious reports on medaka fish.[24,27] The exposure was performed in 5 L tanks filled with 4 L of carbon-filtered water. Half of the water in each tank was replaced daily with fresh carbon-filtered water. After 30 days posthatching (dph), larvae were transferred into 12 L tanks with a continuous supply of water containing EE_2 or the vehicle control. A flow-through system with a 2-fold volume of water flowing through every 24 h was used. After 90 day exposure, fish were anesthetized with tricaine methanesulfonate (MS-222), and the gonads of male fish in each treatment group were isolated for intersex observation by GFP fluorescence, and were then used for tissue section for histopathological observation. Fish samples with unidentified genders were determined for genotypic sex using gene specific primers (5′-GGCCGGGTCCCCGGGTG-3′ and 5′-CTGGTACTGCTGGTAGTTGTG-3′) against the sex determination gene DMY (also termed as Dmrt1Y).[33,34] For the short-term bioassay experiment, medaka larvae collected on the first day after hatching were exposed to EE2, 17β-estradiol (17β-E_2) or 4-nonylphenol (4-NP) for 30 days at different concentrations, and then intersex occurrence was assessed. Exposure conditions were the same as mentioned above.

Chemical Analysis. Actual water concentrations of EE2 and 17β-E_2 for each exposure group were determined using solid-phase extraction (SPE) concentrations combined with UPLC-ESI-MS/MS according to our previous paper,[24] and the details were provided in the Supporting Information (SI). Actual concentrations of chemicals were consistent across the range tested. Correspondingly to the nominal EE_2 exposure concentrations of 0.5, 1, 2, 4, 8, 16, and 32 ng/L, the measured levels were 0.42, 0.75, 1.38, 2.09, 5.80, 8.72, and 21.47 ng/L for the 90-day exposure experiment and 0.33, 0.55, 1.35, 2.14, 4.62, 9.04, and 19.64 ng/L for the 30 day exposure experiment. The measured levels in 17β-E_2 exposure group were 2.97, 6.77, 13.57, 25.61, and 44.80 ng/L corresponding to the nominal

17β-E_2 exposure concentrations of 5, 10, 20, 40, and 80 ng/L. And the measured levels were 2.12, 6.96, 22.51, and 76.74 μg/L corresponding to nominal 4-NP exposure concentrations of 1.56, 6.25, 25, and 100 μg/L. Concentrations of chemicals were below the detection limit for all vehicle control aquaria (<0.1 ng/L for EE_2 and 17β-E_2, < 1.0 μg/L for 4-NP).

Measurement of Intersex. For intersex observation using GFP fluorescence, male fish were anesthetized with MS-222, and the testes were then isolated, placed in a Petri dish, and quickly observed by a fluorescence stereoscopic microscope (Leika M165 FC, Germany), with a 10× objective lens and GFP filter sets. Fluorescent images were recorded by a color digital cooled charge-coupled device (CCD) camera (Leica DFC310 FX, Germany). The intersex incidence was calculated as the number of male individuals with all kind of severities relative to that of males in the exposure group. The measurement of intersex severity index by GFP fluorescence was performed by manually counting the number of primary oocytes in the whole testis of medaka fish. The testis from each fish was then stored in 10% formaldehyde solution until histological intersex determination.

For paraffin tissue section, fixed gonad samples were dehydrated in a graded alcohol series and embedded in paraffin blocks. Tissue sections (5 μm) were taken from each sample in a step-section manner (6−8 sections per fish), mounted on microscope slides, stained with hematoxylin and eosin (H&E) using standard staining techniques, and then examined by light microscopy for routine histological analyses. For frozen section, fixed gonad samples were embedded in Tissue-Tek OCT compound (Sakura Finetek, St, Torrance, CA). After GFP fluorescence observation by the fluorescence stereoscopic microscope (Leika M165 FC, Germany), the frozen section was stained with H&E as described above.

Gene Expression Analysis. For gene expression analysis, gonads from medaka fish at different developmental stages were isolated and frozen in liquid nitrogen until use. Total RNA was extracted from the samples using TRIzol reagent (Invitrogen) according to manufacturer's protocols. To prevent genomic DNA contamination, total RNA was digested by DNase I (TaKaRa Biotechnology) and purified. The RNA concentrations were adjusted to 100 ng/μL for first-strand cDNA synthesis. First-strand cDNA synthesis and quantitative real-time RT-PCR were performed according to methods described previously. Primers for quantification of mRNA abundance were designed using Premier Primer 5.0 (Premier Biosoft International, Palo Alto, CA), and the forward primer for OSP1 gene was designed as intron-spanning primer to minimize the DNA contamination. Primers for OSP1 were 5′-CCCAAG-GAAGTGTCCCAAAT-3′ and 5′-ATAACCACCA-TACTGTCCAGAAGG-3′, and the amplicon size was 116 bp. Primers for GFP were 5′-TGAACCGCATCGAGCT-GAAGGG-3′ and 5′-ATGTTGTGGCGGATCTTGAAG-3′, and the amplicon size was 154 bp. Ribosomal protein L7 (RPL-7), an appropriate endogenous control for gene expression profiling in EDCs studies,[35] was used as the internal control. Relative expression was evaluated by comparative cycle threshold (Ct) as described by Applied Biosystems (Foster City, CA).

RESULTS AND DISCUSSION

Development of Transgenic Medaka Fish. A sequence of 2067 bp NTS upstream of the translation initiation site (ATG) of the OSP1 gene was isolated and characterized (SI Figure 1). Of this sequence, 467 bp was not identified in the medaka genome database (http://www.ensembl.org/Oryzias_latipes/Info/Index). This promoter sequence was then inserted into the pEGFP-1 vector to construct the chimeric plasmid, pMOSP1-EGFP. The linearized pMOSP1-EGFP plasmid was injected into medaka eggs and the embryos were raised to adult stage. Of the 171 founder fish screened, one female and two males were identified as capable of germ-line transmission of the transgenes. The transmission rate was less than 2%. Among 57 F1 larvae collected from the founders used in this study, 15 expressed GFP gene. The germ-line transmission rate from founder to F1 was about 26.3%. From F1 to subsequent generations, standard Mendelian ratios of transgene inheritance were observed, suggesting a single integration of the transgene. The transgenic medaka line was maintained for over three generations, and the pattern of GFP response did not change, indicating that the transgene was stable and the response was consistent over successive generations without a silencing effect.

Through the body surface of the pMOSP1-EGFP transgenic fish, GFP expression was observed only in the postabdomen of female fish (Figure 1Bii), and was not observed in males at all

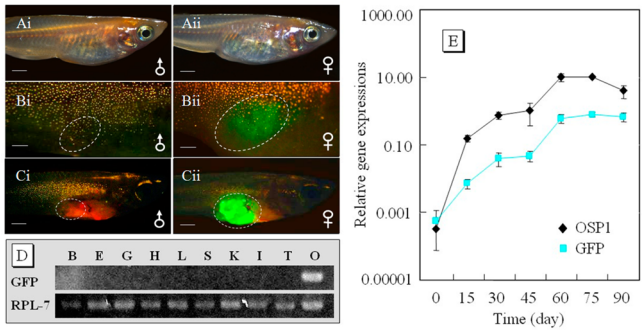


Figure 1. GFP expression in pMOSP1-EGFP transgenic adult medaka. (A-B) Live fish observation of GFP expression in pMOSP1-EGFP transgenic male fish (left) and female fish (right). (C) Gonad observation of GFP expression in abdomen-opened pMOSP1-EGFP transgenic male (left) and female (right). (D) Tissue distribution of GFP expression (brain (B), eye (E), gill (G), heart (H), liver (L), spleen (S), muscle (M), intestine (I), testis (T), and ovary (O)). (E) Variation of OSP1 and GFP mRNA expression levels with developmental stages. Scale bar = 2 mm in (Ai), (Aii), (Ci), (Cii); 1 mm in (Bi), (Bii).

(Figure 1Bi). Since the pigmentation was observed in adult medaka, we dissected female and male medaka fish and found that GFP was expressed specifically in the ovary but not in the testis (Figure 1Ci, Cii). Tissue distribution studies of GFP transcripts using RT-PCR and agarose gel electrophoresis further confirmed that GFP was expressed exclusively in the ovary (Figure 1D), but not in the brain, eye, gill, heart, liver, spleen, muscle, intestine, or testis of adult medaka fish. GFP fluorescence on the body surface of female medaka fish was observed as early as about one week post hatching, and continued to increase during ovarian development, even with overlaying pigmentation in the abdominal cavity. No more apparent changes in GFP fluorescence were observed in females after sexual maturity. The relative expression levels of OSP1 and GFP mRNAs in females at different developmental stages were quantified using real-time RT-PCR. The GFP mRNA expressed in female fish during the early sexual differentiation stage was about 13.1 folds at 15 days post

dx.doi.org/10.1021/es4052796 | Environ. Sci. Technol. 2014, 48, 2413−2420

hatching (dph) compared with that at 0 dph, and the expression level showed a 1093.6-fold increase at sexual maturity (about 60 dph) (Figure 1E). While the absolute expression level of GFP was generally about 10-fold lower than OSP1 mRNA expression, the trend of GFP gene expression with developmental stage almost mimicked the variation in endogenous OSP1 gene expression.

Comparison of Intersex Observation by Transgenic Medaka with Histology. Intersex with different severity indexes in the transgenic medaka fish were induced by EE_2, an environmental estrogen widely detected in water bodies. Since the testis size of medaka fish was small enough for the fluorescence signal emission, the whole testis can be used for the fluorescence observation in the present study, and the primary oocyte with GFP fluorescence could be identified clearly even though these oocytes located deep within the testis. The primary oocytes in the testes, with diameters as small as 10 μm, were accurately distinguished from spermatozoids and other somatic cells by GFP fluorescence observation (Figure 2B); however, these primary oocytes could not be observed by optical microscopy (Figure 2C). A similar phenomenon was also observed in previous results where OSP1 gene expression in some testis samples was induced at low EE_2 exposure concentrations, but primary oocytes could not be detected using light microscopy.[24] This may be due to the fact that the diameters of the observable oocytes as seen by an optical microscope system are usually greater than 20 μm[20,23] or may be due to the limited observation area of testis under an optical microscope system. In addition, frozen-section studies were performed to demonstrate the specificity of GFP expression. As shown in Figure 2B and 2Bi, GFP was expressed exclusively in the primary oocytes, but not in spermatozoids and other somatic cells in the testis of medaka fish under EE2 exposure, which was quite consisted with the transcript locations of OSP1.[24] These results demonstrated that GFP expression restricted to OSP1-expressing cells in the transgenic medaka and can specifically indicate intersex incidence, while the colocalizations of OSP1 and GFP transcripts using methods like double label in situ hybridization should be more direct to confirm this. The transgenic fish used in this study also allowed comprehensive evaluation of primary oocyte distribution in the whole testis. Examination of 52 intersex gonads from fish exposed to 1−8 ng/L EE_2 revealed that while the incidence of primary oocytes was relatively high around the central axis of the testes from a horizontal perspective, the primary oocytes were generally scattered randomly throughout the testicular tissue, and thus the incidence of primary oocytes in testes by optical microscope system would be overlooked.

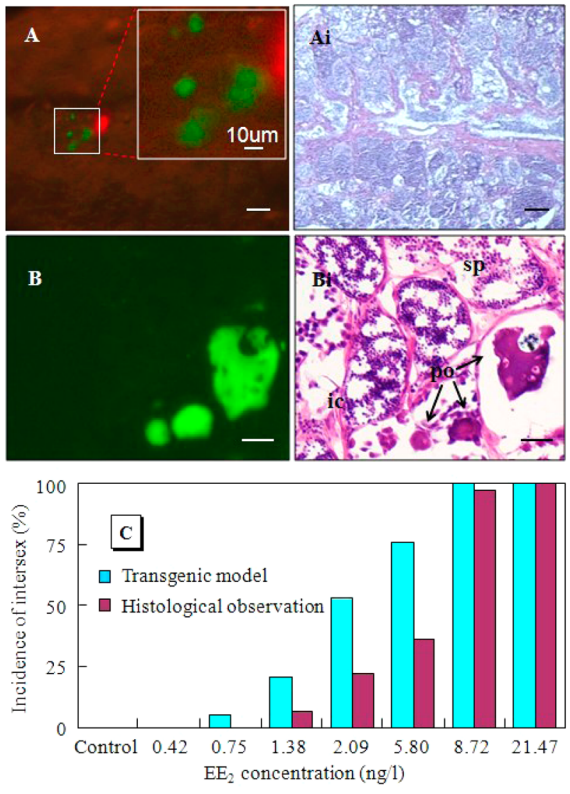


Figure 2. Intersex incidence in transgenic male medaka fish exposed to EE_2 for 3 months (0−90 dph). (A) GFP expression in primary oocytes in testis with intersex; (Ai) Light micrograph of testis in Figure (A) by paraffin slice and H&E staining. (B) GFP expression in primary oocytes (po) but not in spermatozoids (sp) and interstitial somatic cells (ic) in testis with intersex by frozen-section; (Bi) Light micrograph of testis in Figure (B) by H&E staining. Scale bar = 50 μm in (A) and (Ai); 25 μm in (B) and (Bi). (C) Comparison of intersex incidence in male medaka fish measured by the transgenic fish model with traditional histological observation (n = 32−57 for each exposure group).

The intersex incidences of male medaka fish exposed to EE_2 at different concentrations from hatching to 90 dph were 0.0% (0/44), 0.0% (0/57), 5.3% (2/38), 20.5% (9/44), 53.1% (17/32), 75.8% (25/33), 100% (35/35), and 100% (43/43) in the control, 0.5, 1, 2, 4, 8, 16, and 32 ng/L EE_2 exposure groups, respectively (Figure 2A). The lowest EE_2 concentration to induce intersex with an incidence of 5.3% was 1 ng/L (measured as 0.75 ng/L), which was much lower than that in the previous report where the lowest observable effective concentration (LOEC) for EE_2 on the intersex incidence was closer to 2 ng/L.[28] To accurately characterize the intersex severity indexes of male medaka fish in each EE_2 exposure group, the index in individual fish was arbitrarily graded into five levels: 0, no detectable primary oocytes with GFP expression in the testis (Ai); 1, 1−10 primary oocytes observed in the testis (Aii); 2, 11−100 primary oocytes observed in the testis (Aiii); 3, more than 100 primary oocytes observed in the testis (Aiv); 4, sex reversal (Av) (Figure 3A). The intersex severity index was evaluated completely and showed a dose-dependent increase with increasing EE_2 concentrations. The intersex severity indexes in the 1 and 2 ng/L EE_2 exposure groups were all level 1, with intersex incidences of 5.3% (2/38) and 20.5% (9/44) (Figure 3D, E), respectively, and the diameters of most oocytes were about 10 μm. In the 4 ng/L EE_2 exposure group, the intersex severity index ranged from level 1 to 3, with an intersex incidence of 21.9% (7/32), 15.6% (5/32), and 15.6% (5/32), respectively (Figure 3F). In the 8 and 16 ng/L EE_2 exposure groups, the intersex severity indexes ranged widely from level 1 to 4. In the 8 ng/L EE_2 exposure group, the intersex incidences were 33.3% (11/33) for level 1, 24.2% (8/33) for level 2, 12.1% (4/33) for level 3, and 6.1% (2/33) for level 4; and 2.9% (1/35) for level 1, 5.7% (2/35) for level 2, 22.9% (8/35) for level 3, and 68.6% (24/35) for level 4 in the 16 ng/L EE_2 exposure group (Figure 3G, 3H). In the 32 ng/L EE_2 exposure group, all testis samples showed sex reversal (level 4) (Figure 3I). These findings suggested an accurate and sensitive transgenic fish model for assessing both the incidence and severity index of intersex.

dx.doi.org/10.1021/es4052796 | Environ. Sci. Technol. 2014, 48, 2413−2420

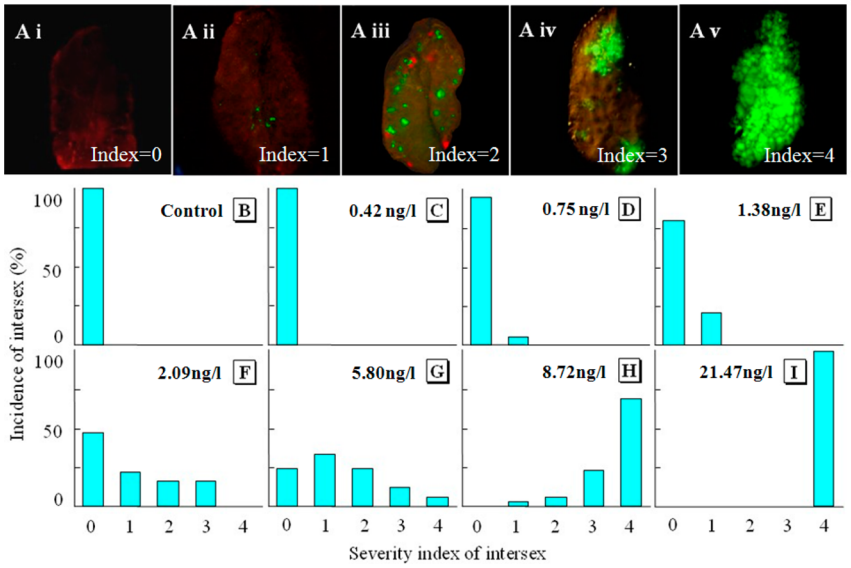


Figure 3. Intersex severity index in transgenic male medaka exposed to EE_2 for 3 months (0−90 dph). (A) Testis with intersex at different severity index in transgenic male medaka. Incidence of intersex with different severity index in control group (B) and each EE_2 exposure group. The number was 44, 57, 38, 44, 32, 33, 35, and 43 for control, 0.42, 0.75, 1.38, 2.09, 5.80, 8.72, and 21.47 ng/L EE_2 exposure groups, respectively.

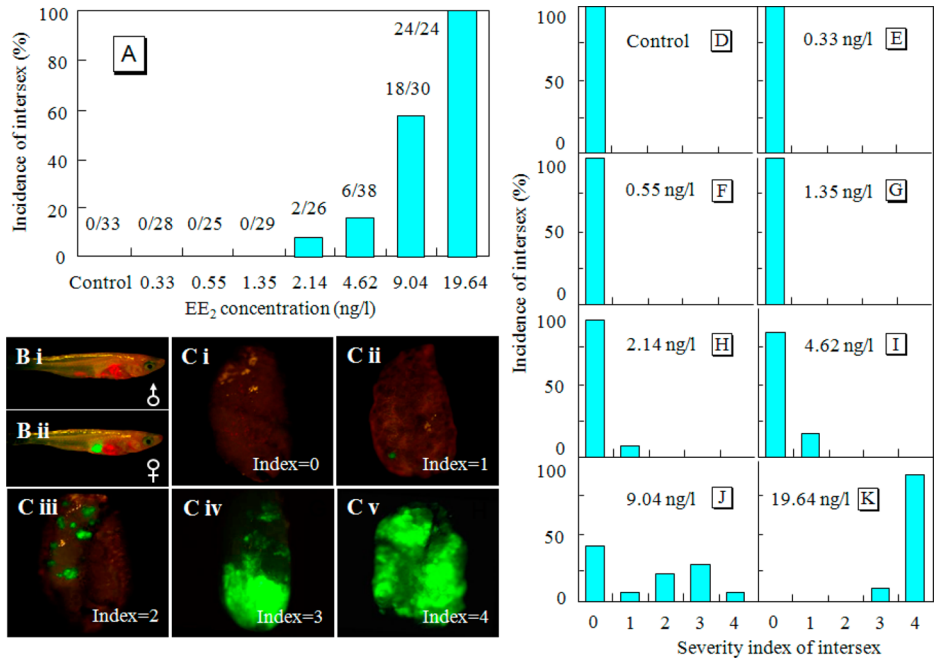


Figure 4. Intersex occurrence in transgenic male medaka fish exposed to EE_2 for 30 days (0−30dph). (A) Incidence of intersex measured by transgenic fish exposed to 0.33−19.64 ng/L EE_2. (B) Gonad observation of GFP expression in abdomen-opened pMOSP1-EGFP transgenic male (top) and female (bottom). (C) Testis at different intersex severity index in transgenic male medaka. Incidence of intersex with different severity index in control group (D) and each EE_2 exposure group.

Short-Term Reporter Assay. Exposure to estrogenic chemicals from hatching to sexual maturity in model fish species has been widely used for histopathological observation of intersex. Such exposure usually lasts a relatively long time, such as 3−6 months for medaka (*Oryzias latipes*).[27,28] The considerable amount of time, labor, and chemicals required for these studies has limited the number of chemicals that can be screened for estrogenic activity using intersex as an end point. It is accepted that early developmental stages tend to be more sensitive to intersex induction than fish in the adult stage.[25,26] However, it was difficult to determine intersex via histological methods in fish at early developmental stages due to the challenge of isolating tissue in small immature testes for sectioning. The transgenic medaka fish developed in the present study provide a tool to characterize intersex appearance in different sex periods in males without complex process for tissue section (one month). Using this short-term assay, we assessed the estrogenic activity of EE_2 in relation to intersex induction. For male medaka fish exposed to different concentrations of EE_2 from hatching to 30 dph, the intersex incidences were 0.0% (0/33), 0.0% (0/28), 0.0% (0/25), 0.0% (0/29), 7.7% (2/26), 15.8% (6/38), 57.0% (18/30), and 100%

dx.doi.org/10.1021/es4052796 | Environ. Sci. Technol. 2014, 48, 2413−2420

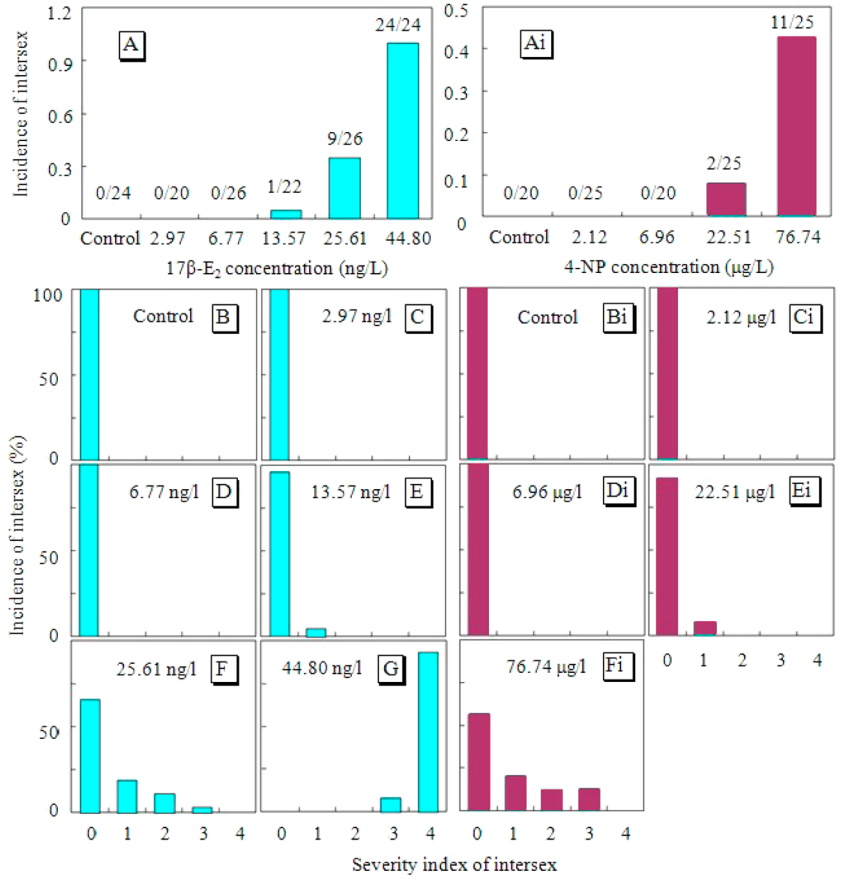


Figure 5. Intersex occurrence in transgenic male medaka fish exposed to 17β-E2 or 4-NP for 30 days (0−30 dph). (A) Incidence of intersex measured by transgenic fish exposed to 2.97−44.80 ng/L 17β-E2. (B-G) Incidence of intersex with different severity index in control group (B) and each 17β-E2 exposure group (C: 2.97 ng/L; D: 6.77 ng/L; E: 13.57 ng/L; F: 25.61 ng/L; G: 44.80 ng/L). (Ai) Incidence of intersex measured by transgenic fish exposed to 2.12−76.74 μg/L 4-NP. (Bi-Fi) Incidence of intersex with different severity index in control group (Bi) and each 4-NP exposure group (Ci: 2.12 μg/L; Di: 6.96 μg/L; Ei: 22.51 μg/L; Fi: 76.74 μg/L).

(24/24) in the control, 0.5, 1, 2, 4, 8, 16, and 32 ng/L EE_2 exposure groups, respectively (Figure 4A), showing a dose-dependent increase. The lowest EE_2 concentration to induce intersex occurrence (7.7%, 2/26) was 4 ng/L (measured as 2.14 ng/L), which was higher than that (1 ng/L) (measured as 0.75 ng/L) in the 3-month exposure assay (Figure 2A). The average diameter of primary oocytes induced by EE_2 was approximately 26.6 ± 5.9 μm, which was also smaller than that (37.3 ± 17.1 μm) in the 3-month exposure assay. This was consistent with earlier research on roach exposure to wastewater effluent,[26] where intersex severity was related to age and possibly to growth stage of primary oocytes over time. In the present study, intersex severity index was determined for each exposure group. Only intersex at level 1 was observed in the 4 and 8 ng/L groups, although intersex incidence was higher in the 8 ng/L group (15.8%) than that in 4 ng/L group (7.7%). For medaka fish exposed to 16 ng/L EE_2, the intersex severity index ranged widely from level 1 to 4, and the corresponding incidences were 6.7%, 20.0%, 26.7%, and 6.7%, respectively, with the distribution of the intersex severity index similar to that in the 8 ng/L group in the 3 month assay. When the concentration of EE_2 increased to 32 ng/L, most testis samples (90.9%) showed sex reversal, similar to that observed in the 3 month assay (Figure 4C).

To further demonstrate the applications of the pMOSP1-EGFP transgenic fish line in the screening of environmental estrogens, we also assessed the estrogenic activities of 17β-E_2 and 4-NP using intersex as the end point. As shown in Figure 5A, the intersex incidences for male medaka fish exposed to 17β-E_2 (0−30 dph) were 0.0% (0/24), 0.0% (0/22), 0.0% (0/26), 4.2% (1/24), 46.2% (12/26) and 100% (24/24) in the control, 5, 10, 20, 40, and 80 ng/L, respectively, showing a dose-dependent increase. The lowest 17β-E_2 concentration to induce intersex with an incidence of 4.2% was 20 ng/L (measured as 13.57 ng/L), which was slightly higher than that in a 100-day 17β-E_2 exposure experiment where the LOEC for 17β-E_2 on the intersex incidence (10.0%) of medaka fish was closer to 10 ng/L.[36] The intersex incidences were 0.0% (0/23), 0.0% (0/25), 0.0% (0/25), 7.1% (2/28), and 58.3% (14/24) in the control, 1.56, 6.25, 25, and 100 μg/L 4-NP exposure groups, respectively (Figure 5Ai). The lowest 4-NP concentration to induce intersex with an incidence of 7.1% was 25 μg/L (measured as 22.51 μg/L), which was similar with the previous results where the LOEC for intersex incidence (4.6%) of medaka fish exposed to 4-NP for more than 3 month (0−100 dph) was about 30 μg/L.[37] Intersex severity index was also determined for each exposure group. For medaka fish exposed to 17β-E_2 at 20 ng/L, only intersex at level 1 was observed with 4.2% incidents, and intersex at level 1 to 4 in the 40 ng/L group were observed with incidences of 19.2%, 11.5%, 7.7%, and 7.7%, respectively. When the concentration of 17β-E_2 increased to 80 ng/L, most testis samples (91.7%) showed sex reversal

dx.doi.org/10.1021/es4052796 | Environ. Sci. Technol. 2014, 48, 2413−2420

(Figure 5B–G). For medaka fish exposed to 4-NP, intersex at level 1 was observed in 25 μg/L group (7.1%), and intersex at levels 1–4, with the corresponding incidences 25.0%, 12.5%, 12.5%, and 4.2%, respectively, was observed in 100 μg/L group (Figure 5Bi-Gi). These findings further demonstrated that the newly developed transgenic medaka fish has the potential to detect the incidence of intersex with shorter exposure time and more rapid and sensitive identification of intersex than current assays. OECD has established some criterions of intersex for assessing estrogenic chemicals in fish based on the histopathological observation by optical microscope, and the exposure period always needs 3–6 months for this histopathological observation of intersex in medaka (OECD Histopathology Guidance Document, 2009). Therefore, it can be expected that the newly developed transgenic medaka fish enhanced capability for the screening of chemicals with potential reproduction toxicity due to intersex incidences. In addition, since natural and man-made estrogens have been well detected in river basin and the discharged effluents of municipal sewage treatment plant at comparable level inducing intersex observed in the present study,[38−41] the newly developed transgenic medaka fish will be useful to in situ detect intersex incidence by mixture chemicals in environmental water.

■ ASSOCIATED CONTENT

Supporting Information

Detailed descriptions of methods for chemicals analysis and DNA sequence of the proximal promoter region of OSP1 are described in the supplementary data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone/fax: 86-10-62765520; e-mail: hujy@urban.pku.edu.cn.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support from the National Natural Science Foundation of China [41330637, 41171385 and 20977002] and Key Projects in the National Science & Technology Pillar Program [2012BAD20B05] is gratefully acknowledged.

■ REFERENCES

(1) Kolpin, D. W.; Furlong, E. T.; Meyer, M. T.; Thurman, E. M.; Zaugg, S. D.; Barber, L. B.; Buxton, H. T. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999–2000: A national reconnaissance. *Environ. Sci. Technol.* **2002**, *36* (6), 1202–1211.

(2) Jobling, S.; Nolan, M.; Tyler, C. R.; Brighty, G.; Sumpter, J. P. Widespread sexual disruption in wild fish. *Environ. Sci. Technol.* **1998**, *32* (17), 2498–2506.

(3) Nash, J. P.; Kime, D. E.; Van der Ven, L.; Wester, P. W.; Brion, F.; Maack, G.; Stahlschmidt-Allner, P.; Tyler, C. R. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environ. Health Perspect.* **2004**, *112* (17), 1725–1733.

(4) Sharpe, R. M.; Skakkebaek, N. E. Are estrogens involved in falling sperm counts and disorders of the male reproductive tract. *Lancet* **1993**, *341* (8857), 1392–1395.

(5) Stone, R. Environmental estrogens stir debate. *Science* **1994**, *265* (5170), 308–310.

(6) Kidd, K. A.; Blanchfield, P. J.; Mills, K. H.; Palace, V. P.; Evans, R. E.; Lazorchak, J. M.; Flick, R. W. Collapse of a fish population after exposure to a synthetic estrogen. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (21), 8897–8901.

(7) OECD Guidance Document on Standardised Test. *Guidelines for Evaluating Chemicals for Endocrine Disruption*, OECD Environment Health and Safety Publications Series on Testing and Assessment No 150 (Draft April 2012). Organisation for Economic Cooperation and Development, Environment, Health and Safety Division: Paris, 2012.

(8) US EPA Endocrine Disruptor Screening Program. Weight-of-evidence: Evaluation results of EDSP Tier 1 screening to identify the need for Tier 2 testing. 2011; http://www.epa.gov/endo/.

(9) OECD Guidelines for the Testing of Chemicals. *Section 4. Test No. 457: BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists*; Organisation for Economic Cooperation and Development, Environment, Health and Safety Division: Paris, 2012.

(10) Ankley, G.; Mihaich, E.; Stahl, R.; Tillitt, D.; Colborn, T.; McMaster, S.; Miller, R.; Bantle, J.; Campbell, P.; Denslow, N.; Dickerson, R.; Folmar, L.; Fry, M.; Giesy, J.; Gray, L. E.; Guiney, P.; Hutchinson, T.; Kennedy, S.; Kramer, V.; LeBlanc, G.; Mayes, M.; Nimrod, A.; Patino, R.; Peterson, R.; Purdy, R.; Ringer, R.; Thomas, P.; Touart, L.; Van der Kraak, G.; Zacharewski, T. Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. *Environ. Toxicol. Chem.* **1998**, *17* (1), 68–87.

(11) Kinnberg, K. *Evaluation of in Vitro Assays for Determination of Estrogenic Activity in the Environment*; Copenhagen, Denmark: Danish Environmental Protection Agency, 2003.

(12) Lee, O.; Takesono, A.; Tada, M.; Tyler, C. R.; Kudoh, T. Biosensor zebrafish provide new insights into potential health effects of environmental estrogens. *Environ Health Perspect.* **2012**, *120* (7), 990–996.

(13) Brion, F.; Le Page, Y.; Piccini, B.; Cardoso, O.; Tong, S. K.; Chung, B. C.; Kah, O. Screening estrogenic activities of chemicals or mixtures in vivo using transgenic (cyp19a1b-GFP) zebrafish embryos. *PLoS One.* **2012**, *7* (5), e36069.

(14) Petersen, K.; Fetter, E.; Kah, O.; Brion, F.; Scholz, S.; Tollefsen, K. E. Transgenic (cyp19a1b-GFP) zebrafish embryos as a tool for assessing combined effects of oestrogenic chemicals. *Aquat Toxicol.* **2013**, *138–139*, 88–97.

(15) Filby, A. L.; Ortiz-Zarragoitia, M.; Tyler, C. R. The *vas::egfp* transgenic zebrafish: A practical model for studies on the molecular mechanisms by which environmental estrogens affect gonadal sex differentiation. *Environ. Toxicol. Chem.* **2013**, DOI: 10.1002/etc.2467.

(16) Takatsu, K.; Miyaoku, K.; Rani Roy, S.; Murono, Y.; Sago, T.; Itagaki, H.; Nakamura, M.; Tokumoto, T. Induction of female-to-male sex change in adult zebrafish by aromatase inhibitor treatment. *Sci. Rep.* **2013**, *3*, 3400 DOI: 10.1038/srep03400.

(17) Jobling, S.; Coey, S.; Whitmore, J. G.; Kime, D. E.; Van Look, K.; McAllister, B. G.; Beresford, N.; Henshaw, A. C.; Brighty, G.; Tyler, C. R.; Sumpter, J. P. Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biol. Reprod.* **2002a**, *67* (2), 515–524.

(18) Jobling, S.; Beresford, N.; Nolan, M.; Rodgers-Gray, T.; Brighty, G. C.; Sumpter, J. P.; Tyler, C. R. Altered sexual maturation and gamete production in wild roach (Rutilus rutilus) living in rivers that receive treated sewage effluents. *Biol. Reprod.* **2002b**, *66* (2), 272–281.

(19) An, W.; Hu, J. Y.; Giesy, J. P.; Yang, M. Extinction risk of exploited wild roach (rutilus rutilus) populations due to chemical feminization. *Environ. Sci. Technol.* **2009**, *43* (20), 7895–7901.

(20) Vigano, L.; Arillo, A.; Bottero, S.; Massari, A.; Mandich, A. First observation of intersex cyprinids in the Po River (Italy). *Sci. Total Environ.* **2001**, *269* (1–3), 189–194.

(21) Hinck, J. E.; Blazer, V. S.; Schmitt, C. J.; Papoulias, D. M.; Tillitt, D. E. Widespread occurrence of intersex in black basses (*Micropterus* spp.) from US rivers, 1995–2004. *Aquat. Toxicol.* **2009**, *95* (1), 60–70.

(22) Johnson R., Wolff J., Braunbeck T. OECD guidance document for the diagnosis of endocrine-related histopathology of fish gonads. *Histopathology 96*, **2009**.

(23) Bjerregaard, L. B.; Korsgaard, B.; Bjerregaard, P. Intersex in wild roach (*Rutilus rutilus*) from Danish sewage effluent-receiving streams. *Ecotoxicol. Environ. Saf.* **2006**, *64* (3), 321−328.
(24) Zhao, Y. B.; Hu, J. Y. Development of a molecular biomarker for detecting intersex after exposure of male medaka fish to synthetic estrogen. *Environ. Toxicol. Chem.* **2012**, *31* (8), 1765−1773.
(25) Koger, C. S.; Teh, S. J.; Hinton, D. E. Determining the sensitive developmental stages of intersex induction in medaka (*Oryzias latipes*) exposed to 17 beta-estradiol or testosterone. *Mar. Environ. Res.* **2000**, *50* (1−5), 201−206.
(26) Liney, K. E.; Jobling, S.; Shears, J. A.; Simpson, P.; Tyler, C. R. Assessing the sensitivity of different life stages for sexual disruption in roach (*Rutilus rutilus*) exposed to effluents from wastewater treatment works. *Environ. Health Perspect.* **2005**, *113* (10), 1299−1307.
(27) Gray, M. A.; Metcalfe, C. D. Induction of testis-ova in Japanese medaka (*Oryzias latipes*) exposed to p-nonylphenol. *Environ. Toxicol. Chem.* **1997**, *16* (5), 1082−1086.
(28) Balch, G. C.; Mackenzie, C. A.; Metcalfe, C. D. Alterations to gonadal development and reproductive success in Japanese medaka (*Oryzias latipes*) exposed to 17 alpha-ethinylestradiol. *Environ. Toxicol. Chem.* **2004**, *23* (3), 782−791.
(29) Foran, C. M.; Peterson, B. N.; Benson, W. H. Transgenerational and developmental exposure of japanese medaka (Oryzias latipes) to ethinylestradiol results in endocrine and reproductive differences in the response to ethinylestradiol as adults. *Toxicol. Sci.* **2002**, *68* (2), 389−402.
(30) Shima, A.; Mitani, H. Medaka as a research organism: Past, present and future. *Mech. Dev.* **2004**, *121* (7−8), 599−604.
(31) Zeng, Z. Q.; Shan, T.; Tong, Y.; Lam, S. H.; Gong, Z. Y. Development of estrogen-responsive transgenic medaka for environmental monitoring of endocrine disrupters. *Environ. Sci. Technol.* **2005**, *39* (22), 9001−9008.
(32) Kurauchi, K.; Nakaguchi, Y.; Tsutsumi, M.; Hori, H.; Kurihara, R.; Hashimoto, S.; Ohnuma, R.; Yamamoto, Y.; Matsuoka, S.; Kawai, S.; Hirata, T.; Kinoshita, M. In vivo visual reporter system for detection of estrogen-like substances by transgenic medaka. *Environ. Sci. Technol.* **2005**, *39* (8), 2762−2768.
(33) Nanda, I.; Kondo, M.; Hornung, U.; Asakawa, S.; Winkler, C.; Shimizu, A.; Shan, Z. H.; Haaf, T.; Shimizu, N.; Shima, A.; Schmid, M.; Schartl, M. A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99* (18), 11778−11783.
(34) Matsuda, M.; Nagahama, Y.; Shinomiya, A.; Sato, T.; Matsuda, C.; Kobayashi, T.; Morrey, C. E.; Shibata, N.; Asakawa, S.; Shimizu, N.; Hori, H.; Hamaguchi, S.; Sakaizumi, M. DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature* **2002**, *417* (6888), 559−563.
(35) Zhang, Z. B.; Hu, J. Y. Development and validation of endogenous reference genes for expression profiling of medaka (*Oryzias latipes*) exposed to endocrine disrupting chemicals by quantitative real-time RT-PCR. *Toxicol. Sci.* **2007**, *95* (2), 356−368.
(36) Metcalfe, C. D.; Metcalfe, T. L.; Kiprassis, Y.; Koenig, B. G.; Khan, C.; Hughes, R. J.; Croley, T. R.; March, R. E.; Potter, T. Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays in Japanese medaka (*Oryzias latipes*). *Environ. Toxicol. Chem.* **2001**, *20*, 297−308.
(37) Balch, G.; Metcalfe, C. Developmental effects in Japanese medaka (*Oryzias latipes*) exposed to nonylphenol ethoxylates and their degradation products. *Chemosphere* **2006**, *62*, 1214−1223.
(38) Zhanlan, Fan; Shimin, Wu; Hong, Chang; Jianying, Hu Behaviors of glucocorticoids, androgens and progestogens in a municipal sewage treatment plant: Comparison to estrogens. *Environ. Sci. Technol.* **2011**, *45*, 2725−2733.
(39) Hong, Chang; Yi, Wan; JianYing, Hu Determination and source apportionment of five classes of steroid hormones in urban rivers. *Environ. Sci. Technol.* **2009**, *43*, 7691−7698.
(40) Zhou, Y.; Zha, J.; Wang, Z. Occurrence and fate of steroid estrogens in the largest wastewater treatment plant in Beijing, China. *Environ. Monit. Assess* **2012**, *184* (11), 6799−6813.
(41) Belfroid, A. C.; Van der horst, A.; Vethaak, A. D.; Schäfer, A. J.; Rijs, G. B. J.; Wegener, J.; Cofino, W. P. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. *Sci. Total Environ.* **1999**, *225* (1−2), 101−108.