Chemosphere 80 (2010) 608–612

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Short Communication

In vitro and *in vivo* estrogenic effects of 17α-estradiol in medaka (*Oryzias latipes*)

Chong Huang, Zhaobin Zhang, Shimin Wu, Yanbin Zhao, Jianying Hu *

College of Urban and Environmental Sciences, Peking University, Beijing 100871, China

ARTICLE INFO

Article history:
Received 4 February 2010
Received in revised form 26 March 2010
Accepted 4 April 2010
Available online 6 May 2010

Keywords:
Vitellogenin
Quantitative real-time RT-PCR
Estrogen receptor
Oryzias latipes

ABSTRACT

17α-Estradiol (17α-E2), the stereoisomer of 17β-estradiol (17β-E2), is considered a weak estrogen in mammals. However, little is known about its estrogenic potency in teleost fish, even though 17α-E2 has been frequently detected in aquatic environment. To investigate the estrogenic activity of 17α-E2, an *in vitro* assay based on the ligand-dependent interaction between medaka estrogen receptor alpha (med-ERα) and coactivator was conducted. Japanese medaka (*Oryzias latipes*) were exposed to 1, 10, 100, 1000 and 10 000 ng L^{-1} 17α-E2 (actual concentrations of 1.91, 12.81, 96.24, 1045.15, and 9320.81 ng L^{-1}, respectively) for 3 weeks, and expression for vitellogenins (VTG-I), Choriogenin H (CHG-H) and estrogen receptor α (ERα) mRNA in the livers was analyzed by quantitative real-time RT-PCR (Q-RT-PCR). The *in vitro* study showed that the EC_{50} of 17α-E2 was 114.10 nM, which was 30 times less potent than that of 17β-E2 (3.80 nM). Dose-dependent induction of gene expression of VTG-I, CHG-H and ERα were observed. The benchmark dose (BMD) values for VTG-I, CHG-H and ERα were 44.16 ng L^{-1}, 15.25 ng L^{-1} and 66.03 ng L^{-1}, which were about 11-, 17- and 14-times less potent than that of 17β-E2, respectively. Comparing this study with those reported previously in mammals, it is suggested that fish species may be more susceptive to 17α-E2 than mammals.

1. Introduction

In the last two decades, public concern regarding the feminizing effects of estrogenic chemicals in wild life has been increasing (Jobling et al., 1998). These estrogenic chemicals include natural steroid estrogens, mainly 17β-Estradiol (17β-E2), estrone (E1), estriol (E3) and pharmaceutical estrogens, such as ethinylestradiol (EE2), in addition to some industrial chemicals, such as bisphenol A (BPA), 4-tert-octylphenol (4-t-OP), and 4-nonylphenol (4-NP).

17α-Estradiol (17α-E2) is a natural steroid estrogen with the difference in the steric position of the hydroxy-group on C17 (Fig. 1). It is the predominant estrogen in several ungulates and rodents and has been found in the blood and urine of various other animal species (Gwilliam et al., 1974). In addition, 17α-E2 has been detected in effluents and in aquatic environments such as dairy wastewater (2000 ng L^{-1}) and swine wastewater (680 ng L^{-1}), sewage treatment plants (0.1–9.5 ng L^{-1} in the effluent and 17–30 ng L^{-1} in the influent), and rivers (0.02–0.91 ng L^{-1} in Beijing) (Belfroid et al., 1999; Furuichi et al., 2006; Sarmah et al., 2006; Chang et al., 2009; Miege et al., 2009). While its presence in the environment has received some attention, there is little toxicological information regarding 17α-E2 in fish.

Although 17α-E2 is considered as the biologically inactive stereoisomer of 17β-E2, previous studies have shown that it has weak estrogenic activity. An *in vitro* study showed that 17α-E2 was 500 times less potent than 17β-E2, based on the results of the human estrogen receptor-mediated chemical-activated luciferase gene expression assay (ER-CALUX) (Legler et al., 2002). In primary *Xenopus laevis* hepatocyte cultures, the concentration of 17α-E2 required to cause a significant induction of VTG was 345 times greater than that of 17β-E2 (Mitsui et al., 2007). However, when using the two-hybrid yeast assay, the estrogenic activity of 17α-E2 with the rainbow trout estrogen receptor (rtER) was about 27 times less potent than that of 17β-E2 (Le Guevel and Pakdel, 2001). While these results demonstrate that 17α-E2 may have greater estrogenic activity in fish than in mammals, to date there is no information on the potential estrogenic effects of 17α-E2 for fish *in vivo*.

In the present paper, the agonistic potential of 17α-E2 at the medaka ERα (med-ERα) was measured by a two-hybrid yeast assay. The estrogenic effects of 17α-E2 on medaka were investigated by analyzing expression levels of VTG-I, CHG-H and ERα in the livers of male medaka exposed to different concentrations of 17α-E2 and 17β-E2.

2. Materials and methods

2.1. Chemicals

The 17α-E2 (purity: ⩾98%) and 17β-E2 (purity: ⩾98%) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA) and were

* Corresponding author. Tel./fax: +86 10 62765520.
E-mail address: hujy@urban.pku.edu.cn (J. Hu).

0045-6535/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2010.04.010

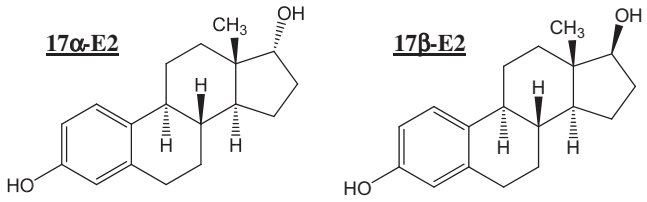

Fig. 1. Chemical structures of 17α-E2 and 17β-E2.

dissolved in dimethyl sulfoxide (DMSO) as the stock solution (stored at −20 °C). All chemicals and reagents used in this study were of molecular biology grade unless otherwise described.

2.2. Yeast two-hybrid assay

The agonist activity of 17α-E2 was examined using a yeast two-hybrid assay with yeast cells (*Saccharomyces cerevisiae* Y190) into which med-ERα and coactivator had been introduced (Nishikawa et al., 1999). Twelve dilutions of 17α-E2 (0.07–12345 nM) and 17β-E2 (0.006–1000 nM) were prepared as exposure concentrations. 17β-E2 was used as a positive control, and DMSO was used as a negative control. All experiments were performed in triplicate (two replicates for each concentration). A detailed description is provided in the supplementary material.

2.3. Experimental animal and chemical exposure

Adult Japanese medaka (*Oryzias latipes*), orange red strain, male, 14-weeks-old, were cultured in flow-through tanks under a constant 16-h light/8-h dark photoperiod and fed with live brine shrimp (*Artemia nauplii*) twice a day. Water used in the experiment was active carbon-treated with a hardness of 8.1 ± 0.1 mg L^{-1} $CaCO_3$, pH 7.7 ± 0.2, dissolved oxygen 7.8 ± 0.3 mg L^{-1} at 25 ± 1 °C. The medaka were acclimated to test water in 10 L tanks for 2 weeks prior to initiation of exposure. After the acclimation period, the 12 male medaka per glass tank were exposed in a flow-through exposure with a final volume of 10 L and complete water renewal every 6 h. Each of the 1, 10, 100, 1000, 10 000 ng L^{-1} 17α-E2 and 0.1, 1, 10, 100, 1000 ng L^{-1} 17β-E2 exposure groups, or vehicle control (0.005% DMSO) was separately exposed in a single treatment tank. Concentrations of 17α-E2 and 17β-E2 in the water of the exposure groups were measured by LC–ESI-MS/MS according to the analytical methods provided in the supplementary material. The method detection limits (MDLs) of 17α-E2 and 17β-E2 for 2 L water samples were 0.05 ng L^{-1} and 0.08 ng L^{-1}, respectively. Concentrations of 17α-E2 and 17β-E2 were found to be below the detection limit in the vehicle control aquaria. Actual concentrations of 17α-E2 were found to be 1.91 ± 1.71, 12.81 ± 5.54, 96.24 ± 18.30, 1045.15 ± 74.79, and 9320.81 ± 151.04 ng L^{-1} for the 1, 10, 100, 1000 and 10 000 ng L^{-1} nominal concentrations, respectively. Actual concentrations of 17β-E2 were found to be 0.49 ± 0.15, 1.13 ± 0.29, 11.79 ± 2.00, 97.49 ± 20.49, and 1038.82 ± 70.75 ng L^{-1} for the 0.1, 1, 10, 100 and 1000 ng L^{-1} nominal concentrations, respectively. At the end of the 21 *d* exposure period, six males were randomly sampled from each of the groups for gene expression analysis. The fish were anaesthetized in ice cold water and killed, and the livers were frozen immediately in liquid nitrogen until the RNA was isolated.

2.4. Total RNA extraction and reverse-transcription PCR

The RNA was extracted from the livers of 66 animals, 6 individuals from each of the exposed groups and the control group. Tissues were thoroughly homogenized before RNA extraction with a glass homogenizer. Total RNA from 10 mg of frozen liver sample was extracted using 1 mL of Trizol (Invitrogen, Life Technologies, USA), according to the manufacturer's instructions. To prevent genomic DNA contamination, the total RNA was digested by DNase I (TaKaRa Biotechnology, China) and then purified. The RNA was then stored at −80 °C until further processing. First-strand cDNA synthesis was performed using Superscript III first-strand synthesis SuperMix and oligo (dT) primers (Invitrogen, Life Technologies, USA). Syntheses of first-strand cDNA were performed according to the methods described previously (Zhang and Hu, 2007).

2.5. Quantitative real-time RT-PCR assay

Gene expression in the livers of medaka was studied using previously established methods (Zhang and Hu, 2008). The PCR primer sequences used for the quantification of the genes encoding VTG-I, CHG-H and ERα are shown in Table 1.

Quantitative real-time PCR (Q-RT-PCR) with SYBR Green detection was performed by using an ABI Prism® 7000 Sequence Detection System (Applied Biosystems, USA) according to protocols established by the manufacturer. The PCR reaction mixture contained 10 μL of 2 × SYBR® Green PCR master mix (Applied Biosystems, USA), 250 nM each of forward and reverse primers, 5 μL of cDNA template, and nuclease-free water in a total volume of 20 μL. The reactants were incubated at 50 °C for 2 min to activate the uracil N9-glycosylase (in SYBR® Green PCR master mix) and then for 10 min at 95 °C to inactivate the uracil N9-glycosylase and activate the Amplitaq Gold® DNA polymerase (in SYBR® Green PCR master mix), followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Following the final cycle of the PCR, the reactions were denatured over a 35 °C temperature gradient at 0.03 °C/s from 60 to 95 °C to determine the quality of PCR products. The quantification of target gene expression was based on the comparative cycle threshold (*Ct*) method. Ribosomal protein L-7 (RPL-7) was used as the internal control (Zhang and Hu, 2007), and relative expression was evaluated by the methods described by Applied Biosystems (Foster City, USA).

2.6. Data analysis

Estrogenic agonist activity of 17α-E2 was recorded as the 50% effective concentration (EC_{50}), which was calculated using non-linear regression according to the dose–response curve by the Prism 4 for Windows program (GraphPad Software, Inc.). The inverse of the EC_{50} value for 17β-E2 was set to 1. For 17α-E2, relative potency (RP), calculated by the ratio of the EC_{50} of 17β-E2 to that of 17α-E2, was used to present its estrogenic activity. For the *in vivo* assay,

Table 1
Sequence of primer for quantitative real-time PCR.

Gene symbol	Gene full name	Accession no.	Sequences (first row, forward primers; second row, reverse primers)	Amplicon size (bp)
RPL-7	Ribosomal protein L7	DQ118296	5′-CGCCAGATCTTCAACGGTGTAT-3′ 5′-AGGCTCAGCAATCCTCAGCAT-3′	72
VTG-I	Vitellogenin I	AB064320	5′-CTCCAGCTTTGAGGCCATTTAC-3′ 5′-ACAGCACGGACAGTGACAACA-3′	81
CHG-H	Choriogenin H	AF500195	5′-TACTTTCCCGTCACTTATTGC-3′ 5′-TTCCACGACCAGAGTTTCAAC-3′	189
ERα	Estrogen receptor alpha	D28954	5′-TTCCACGACCAGAGTTTCAAC -3′ 5′-CCTCTCCCCACCTCGCCTCTC-3′	97

gene expression was calculated as fold induction relative to the average expression in the solvent control group. Benchmark dose (BMD) values (95% confidence interval lower bound) corresponding to a default response (10%) of 17α-E2 and 17β-E2 were calculated by using the USEPA's benchmark dose software version 2.1, with the actual concentrations of 17α-E2 and 17β-E2 determined by LC–ESI-MS/MS. The statistical program SPSS (version 11.5; Chicago, IL, USA) was used to compare and analyze all the collected data. Differences were evaluated by ANOVA followed by Tukey's test. The difference was considered significant at $P < 0.05$.

3. Results and discussion

3.1. In vitro estrogenic activity of 17α-E2: comparison with 17β-E2

Using the yeast two-hybrid assay, the med-ERα agonist activity of 17α-E2 was compared with that of 17β-E2 (Fig. 5). The EC_{50} values of 17α-E2 and 17β-E2 were calculated to be 114.10 nM and 3.80 nM, respectively. Results showed that 17α-E2 was approximately 30 times less potent than 17β-E2. The RP of 17α-E2 to 17β-E2 was estimated to be 0.03, which was similar to that obtained by the rtER yeast assay (Le Guevel and Pakdel, 2001). Agonist rtER activity of 17α-E2 was approximately 27 times less potent than that of 17β-E2. However, hERα agonist activity of 17α-E2 was reported to be over 100 times less potent than that of 17β-E2. Kawagoshi et al. (2002) reported the RP of 17α-E2 to 17β-E2 (set as 1) was 0.01 in a yeast two-hybrid assay incorporating hERα, and Legler et al. (2002) reported that 17α-E2 was 500 times less potent than that of 17β-E2 using an ER-CALUX assay with human T47D breast cells. In addition, the RP of BPA relative to 17β-E2 (set as 1) was estimated to be 3.00×10^{-4} by using the med-ERα yeast assay (Terasaki et al., 2005), indicating that 17α-E2 was approximately 100 times more potent than BPA.

3.2. In vivo transcriptional variation of estrogen-responsive biomarker genes by 17α-E2: comparison with 17β-E2

In this study, dose-dependent up-regulation of VTG-I was observed in the liver of the fish exposed to 17α-E2 and 17β-E2 (Fig. 2). Statistically significant increase in the VTG-I gene expression occurred at concentrations of 100 ng L^{-1} and greater 17α-E2 and 17β-E2 exposure groups ($P < 0.05$). Comparing the VTG-I mRNA expression levels in the exposure groups at highest concentrations of both chemicals, it was found that the VTG-I mRNA expression levels of 17α-E2 was 5184.66 folds which was 2 times lower than that of 17β-E2 (17769.95 folds). Methods of dose–response assessment, such as BMD, have been applied to calculate the RP in many assay systems (Haws et al., 2006). In this study, the BMDs of 17α-E2 and 17β-E2 for VTG-I induction according to the dose–response relationships were calculated to be 44.16 ng L^{-1} and 4.02 ng L^{-1}, and 17α-E2 was about 11 times less potent than 17β-E2 in estrogenic effects. The RP of 17α-E2, a ratio of $BMD_{17\beta\text{-}E2}$ to $BMD_{17\alpha\text{-}E2}$ was calculated to be 0.09. By analyzing VTG induction in the livers of male medaka, Yamaguchi et al.

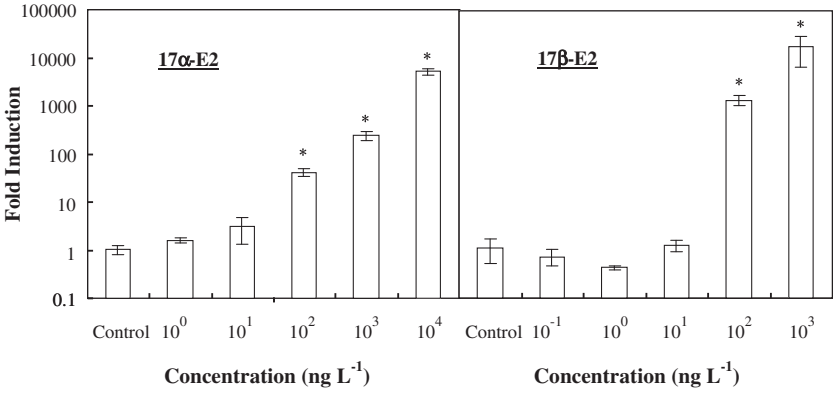

Fig. 2. Relative gene expression of VTG-I in the livers of medaka from the solvent control (0.005% DMSO), 17α-E2 (1, 10, 100, 1000, and 10,000 ng L^{-1}), the positive control of 17β-E2 (0.1, 1, 10, 100, and 1000 ng L^{-1}) by quantitative real-time RT-PCR SYBER Green method ($n = 6$, mean ± SD), asterisk (*) indicates statistically different from control ($P < 0.05$).

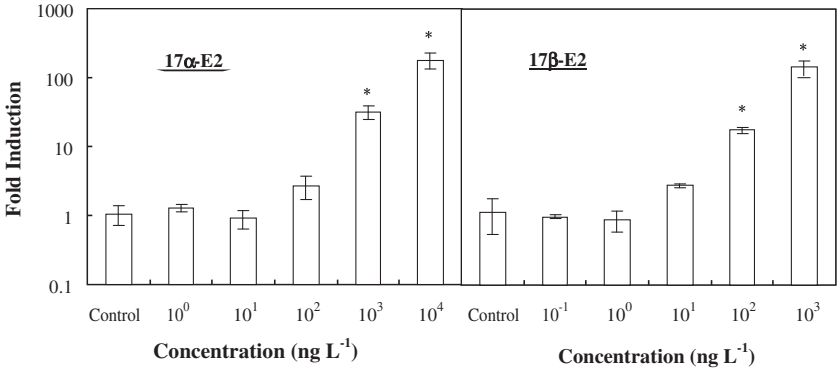

Fig. 3. Relative gene expression of CHG-H in the livers of medaka from the solvent control (0.005% DMSO), 17α-E2 (1, 10, 100, 1000, and 10,000 ng L^{-1}), the positive control of 17β-E2 (0.1, 1, 10, 100, and 1000 ng L^{-1}) by quantitative real-time RT-PCR SYBER Green method ($n = 6$, mean ± SD), asterisk (*) indicates statistically different from control ($P < 0.05$).

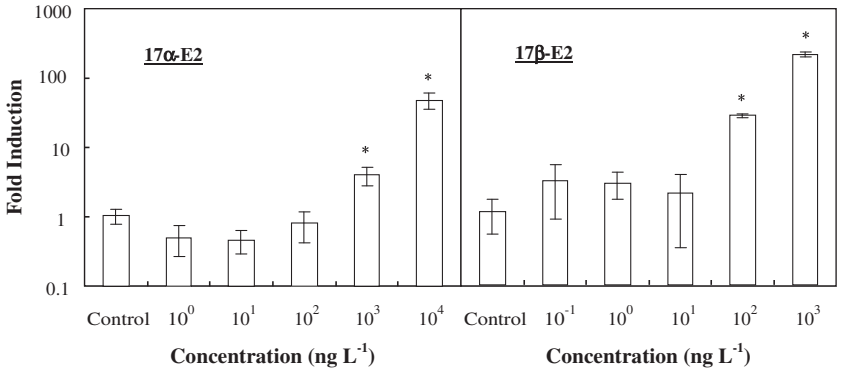

Fig. 4. Relative gene expression of ERα in the livers of medaka from the solvent control (0.005% DMSO), 17α-E2 (1, 10, 100, 1000, and 10,000 ng L^{-1}), the positive control of 17β-E2 (0.1, 1, 10, 100, and 1000 ng L^{-1}) by quantitative real-time RT-PCR SYBER Green method ($n = 6$, mean ± SD), asterisk (∗) indicates statistically different from control ($P < 0.05$).

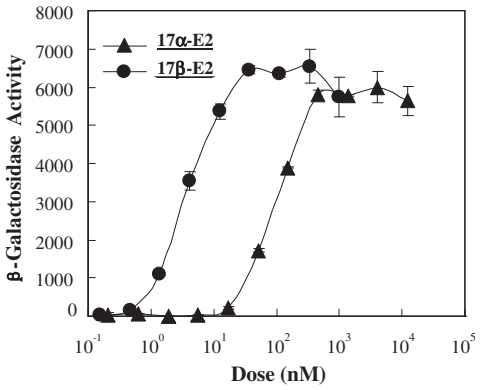

Fig. 5. Estrogenic activities of 17α-E2 and 17β-E2 in yeast two-hybrid assay incorporating medaka estrogen receptor α (med-ERα). Each point represents the mean of three independent experiments, each performed in duplicate.

(2005) reported the estrogenic activities of 4-NP and BPA relative to 17β-E2 (set as 1) to be 0.0002 and 0.00001, respectively. Compared with 4-NP and BPA, 17α-E2 is 450 and 9000 times more potent in inducing VTG expression, although 4-NP and BPA have been given more attention in the previous studies.

CHG-H is a precursor protein of egg envelope of medaka and synthesized in the adult female liver in response to estrogen. It has also been frequently used as a biomarker for monitoring estrogenic EDCs in the aquatic environment (Sugiyama et al., 1999). We measured the CHG-H mRNA level and found that it was significantly ($P < 0.05$) up-regulated in 1000 and 10,000 ng L^{-1} 17α-E2 and 100 and 1000 ng L^{-1} of 17β-E2 exposure groups (Fig. 3). The BMDs of 17α-E2 and 17β-E2 for CHG-H were calculated to be 15.25 ng L^{-1} and 0.88 ng L^{-1}. The estrogenic potency of 17α-E2 was about 17 times lower than that of 17β-E2.

The expression levels of the ERα mRNA in the livers of the male medaka were also assessed. The relative expression levels of ERα mRNA were significantly up-regulated in the livers of the fish exposed to 1000 and 10,000 ng L^{-1} 17α-E2 (actual concentration: 1045.15 and 9320.81 ng L^{-1}) and 100 and 1000 ng L^{-1} 17β-E2 (actual concentration: 97.49 and 1038.82 ng L^{-1}) exposure groups ($P < 0.05$) (Fig. 4). According to their dose–response relationships, the BMD values of 17α-E2 and 17β-E2 for ERα were calculated to be 66.0 ng L^{-1} and 4.9 ng L^{-1}, respectively. The ratio of $BMD_{17\alpha\text{-E2}}$ to $BMD_{17\beta\text{-E2}}$ indicated that 17α-E2 was about 14 times less potent than 17β-E2 in estrogenic effects, which was close to the calculated ratios reported for VTG-I and CHG-H induction. Previous research has shown ERα to be the dominant estrogen receptor in the livers of medaka and it is involved in activating estrogen-responsive genes such as VTG and CHG (Scholz et al., 2004). The up-regulation of ERα observed in the 17α-E2 exposure groups suggested that there would be more ERα in the liver, which could be bound by internal or external estrogenic chemicals and result in an increase in the feminization effect. Considering that 17α-E2 has been frequently detected in aquatic environments (Table S2, in the supplementary material) where fish live their whole life, and it has been demonstrated in fish 17α-E2 was 450 and 9000 times more potent than 4-NP and BPA, two chemicals of current concern regarding its estrogenic properties, respectively. Further attention and research is therefore required on the estrogenic effects of 17α-E2 on fish.

4. Conclusion

In the *in vitro* med-ERα yeast two-hybrid assay, it was found that the estrogenic activity of 17α-E2 was 30 times less potent than that of 17β-E2. In the *in vivo* medaka test, it was shown that 17α-E2 was about 11–17 times less potent than 17β-E2 in inducing VTG-I, CHG-H, and ERα gene expression in livers of male medaka. These results indicate that the RP of 17α-E2 to 17β-E2 in medaka was higher than that reported previously in mammals, and the estrogenic activity of 17α-E2 was much higher than those of some traditional EDCs. More attention should be paid to the effects of 17α-E2 on fish in the future studies.

Acknowledgements

Financial supports from National Natural Science Foundation of China (40632009) and National Basic Research Program of China (2007CB407304) is gratefully acknowledged.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2010.04.010.

References

Belfroid, A.C., Van der Horst, A., Vethaak, A.D., Schafer, A.J., Rijs, G.B.L., Wegener, J., Cofino, W.P., 1999. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in the Netherlands. Sci. Total Environ. 225, 101–108.

Chang, H., Wan, Y., Hu, J.Y., 2009. Determination and source apportionment of five classes of steroid hormones in urban rivers. Environ. Sci. Technol. 43, 7691–7698.

Furuichi, T., Kannan, K., Suzuki, K., Tanaka, S., Giesy, J.P., Masunaga, S., 2006. Occurrence of estrogenic compounds in and removal by a swine farm waste treatment plant. Environ. Sci. Technol. 40, 7896–7902.

Gwilliam, C., Paquet, A., Williams, D.G., Layne, D.S., 1974. Isolation from rabbit urine of 3-glucuronide-17-N-acetyglucosaminide of 17alpha-estradiol. Can. J. Biochem. Cell B 52, 452–455.

Haws, L.C., Su, S.H., Harris, M., DeVito, M.J., Walker, N.J., Farland, W.H., Finley, B., Birnbaum, L.S., 2006. Development of a refined database of mammalian relative potency estimates for dioxin-like compounds. Toxicol. Sci. 89, 4–30.

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

Kawagoshi, Y., Tsukagoshi, Y., Fukunaga, L., 2002. Determination of estrogenic activity in landfill leachate by simplized yeast two-hybrid assay. J. Environ. Monitor. 4, 1040–1046.

Le Guevel, R., Pakdel, F., 2001. Assessment of estrogenic potency of chemicals used as growth promotor by in-vitro methods. Hum. Reprod. 16, 1030–1036.

Legler, J., Jonas, A., Lahr, J., Vethaak, A.D., Brouwer, A., Murk, A.J., 2002. Biological measurement of estrogenic activity in urine and bile conjugates with the *in vitro* ER-CALUX reporter gene assay. Environ. Toxicol. Chem. 21, 473–479.

Miege, C., Karolak, S., Gabet, V., Jugan, M.L., Oziol, L., Chevreuil, M., Levi, Y., Coquery, M., 2009. Evaluation of estrogenic disrupting potency in aquatic environments and urban wastewaters by combining chemical and biological analysis. Trac-Trend. Anal. Chem. 28, 186–195.

Mitsui, N., Tooi, O., Kawahara, A., 2007. Vitellogenin-inducing activities of natural, synthetic, and environmental estrogens in primary cultured *Xenopus laevis* hepatocytes. Comp. Biochem. Phys. C. 146, 581–587.

Nishikawa, J., Saito, K., Goto, J., Dakeyama, F., Matsuo, M., Nishihara, T., 1999. New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. Toxicol. Appl. Pharm. 154, 76–83.

Sarmah, A.K., Northcott, G.L., Leusch, F.D.L., Tremblay, L.A., 2006. A survey of endocrine disrupting chemicals (EDCs) in municipal sewage and animal waste effluents in the Waikato region of New Zealand. Sci. Total Environ. 355, 135–144.

Scholz, S., Kordes, C., Hamann, J., Gutzeit, H.O., 2004. Induction of vitellogenin *in vivo* and *in vitro* in the model teleost medaka (*Oryzias latipes*): comparison of gene expression and protein levels. Mar. Environ. Res. 57, 235–244.

Sugiyama, H., K, Murata, Iuchi, I., Nomura, K., Yamagami, K., 1999. Formation of mature egg envelope subunit proteins from their precursors (choriogenins) in the fish, *Oryzias latipes*: Loss of partial C-terminal sequences of the choriogenins. J. Biochem. 125, 469–475.

Terasaki, M., Shiraishi, F., Nishikawa, T., Edmonds, J.S., Morita, M., Makino, M., 2005. Estrogenic activity of impurities in industrial grade Bisphenol A. Environ. Sci. Technol. 39, 3703–3707.

Yamaguchi, A., Ishibashi, H., Kohra, S., Arizono, K., Tominaga, N., 2005. Short-term effects of endocrine-disrupting chemicals on the expression of estrogen-responsive genes in male medaka (*Oryzias latipes*). Aquat. Toxicol. 72, 239–249.

Zhang, Z.B., Hu, J.Y., 2007. 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. 95, 356–368.

Zhang, Z.B., Hu, J.Y., 2008. Effects of p,p′-DDE exposure on gonadal development and gene expression in Japanese medaka (*Oryzias latipes*). J. Environ. Sci. 20, 347–352.