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Evaluation of Estrogenic Activity of Wastewater: Comparison Among In Vitro ERα Reporter Gene Assay, In Vivo Vitellogenin Induction, and Chemical Analysis

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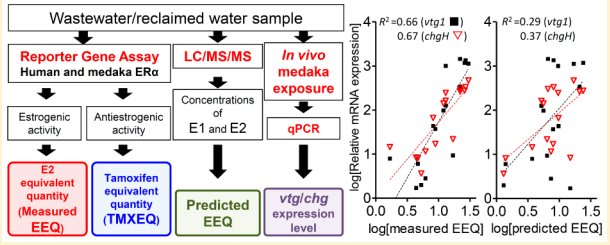
Supporting Information

ABSTRACT: The in vitro estrogen receptor (ER) reporter gene assay has long been used to measure estrogenic activity in wastewater. In a previous study, we demonstrated that the assay represents net estrogenic activity in the balance between estrogenic and antiestrogenic activities in wastewater. However, it remained unclear whether the net estrogenic activity measured by the in vitro ERα reporter gene assay can predict the in vivo estrogenic effect of wastewater. To determine this, we measured the following: estrogenic and antiestrogenic activities of wastewater and reclaimed water by the in vitro ERα reporter gene assay, expression of *vitellogenin-1* (*vtg1*) and *choriogenin-H* (*chgH*) in male medaka (*Oryzias latipes*) by quantitative real-time PCR, and estrone, 17β-estradiol, estriol, and 17α-ethynylestradiol concentrations chemically to predict estrogenic activity. The net estrogenic activity measured by the in vitro medaka ERα reporter gene assay predicted the in vivo *vtg1*/*chgH* expression in male medaka more accurately than the concentrations of estrogens. These results also mean that in vivo *vtg1*/*chgH* expression in male medaka is determined by the balance between estrogenic and antiestrogenic activities. The in vitro medaka ERα reporter gene assay also predicted in vivo *vtg1*/*chgH* expression on male medaka better than the human ERα reporter gene assay.



INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are exogenous substances that alter the function of the endocrine system, with adverse health effects on organisms or their progeny.[1] Effluent of wastewater treatment plants (WWTPs) is one of the major sources of EDCs in the aquatic environment.[2,3] Exposure to WWTP effluent or environmental estrogens has been associated with the induction of vitellogenin in male fish and with intersex in wild fish species.[4−7]

The rapid, highly sensitive, cost-effective in vitro estrogen receptor-alpha (ERα) reporter gene assay is used to measure estrogenic activity in wastewater as an alternative to in vivo testing. For environmental monitoring, it is critical to know how accurately it can predict in vivo estrogenic effects. In general, it has been believed that the assay, which can detect both known and unknown EDCs, can measure the total estrogenic activity in wastewater, and is therefore more accurate at predicting in vivo estrogenic effects than chemical analysis, which can measure only known EDCs. Where measured estradiol equivalent quantities (EEQs) were higher than predicted EEQs based on the concentrations of EDCs detected by chemical analysis, it was speculated that other estrogenic compounds besides the target EDCs were present.[8,9] Conversely, where measured EEQs were lower than predicted, it was speculated that antiestrogenic compounds were

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Table 1. Sample Characteristics and Concentrations of Estrogens, Predicted EEQs, and Measured EEQs[a]

	specifications		concentration (ng/L)[b,c]		predicted EEQ (ng-E2/L)[d]		measured EEQ (ng-E2/L)	
	date	type	E1	E2	medaka ERα	human ERα	medaka ERα	human ERα
expt 1	Dec 2011	SE	56.6	2.5	7.4	3.5	5.3	3.7
		coag + UF	48.6	1.3	5.5	2.2	11	1.4
expt 2	May 2012	SE	61.7	4.2	9.6	5.3	13	5.2
		coag + UF	50.6	2.2	6.6	3.1	22	2.7
		UF	50.3	3.7	8.1	4.6	27	4.0
expt 3	Aug 2012	SE	11.1	2.8	3.8	3.0	<LOD[e]	1.3
		coag + UF	9.8	1.1	2.0	1.3	4.6	1.2
		UF	5.3	0.8	1.3	0.90	5.3	1.7
		UF + RO	ND	ND			<LOD[e]	<LOD[e]
expt 4	Oct 2012	SE	84.0	2.3	9.6	3.8	8.8	5.4
		UF	41.0	1.6	5.2	2.3	13	5.5
		UF + RO	ND	ND			<LOD[e]	<LOD[e]
expt 5	June 2013	SE	135.1	9.5	21	12	22	10
		coag + UF	127.1	12.6	24	15	26	14
		UF	109.0	7.6	17	9.6	30	12
		UF + RO	ND	ND			<LOD[e]	<LOD[e]
expt 6	Aug 2013	SE	84.6	7.2	15	8.7	4.4	8.0
		coag + UF	64.6	2.3	7.9	3.5	8.9	4.8
		UF	48.6	2.1	6.3	3.0	17	5.8
		UF + RO	ND	ND			<LOD[e]	<LOD[e]

[a]SE: secondary effluent; coag + UF: coagulation followed by ultrafiltration; UF: UF alone; UF + RO: UF followed by RO membrane filtration; ND = not detected. [b]E3 and EE2 were not detected. [c]Limits of detection (ng/L): E1, 0.3; E2, 0.5; E3, 0.5; EE2, 0.5. [d]Relative potency values of E1 were cited from our previous study (8.74% for medaka ERα, 1.82% for human).[9] [e]Limits of detection (LOD). Values are indicated in Figure 1.

present.[10−12] The results of our previous study support these speculations, and we concluded that the in vitro ERα reporter gene assay represents net estrogenic activity in the balance between estrogenic and antiestrogenic activities.[9] Therefore, to what extent can the net estrogenic activity measured by the assay predict in vivo estrogenic effects in wastewater? Even when there is a large discrepancy between measured EEQ based on the assay and predicted EEQ based on chemical analysis, is the in vitro ERα reporter gene assay still a reliable predictor of in vivo estrogenic effects in wastewater? For example, if fish are exposed to wastewater in which measured EEQ based on the assay is much higher than predicted EEQ based on the concentrations of natural estrogens, is vitellogenin induced in direct proportion to measured EEQ? Or if fish are exposed to wastewater in which predicted EEQ is high but measured EEQ is lower than predicted because of the presence of antiestrogenic compounds, is vitellogenin not induced? Some studies reported that in vitro methods for detecting estrogenic activity in the aquatic environment could predict in vivo estrogenic effects.[12−15] However, our questions have not been tested.

Here, we investigated whether the measured EEQ based on the in vitro ERα reporter gene assay is a reliable predictor of the induction of in vivo *vtg1*/*chgH* expression. We used the in vitro ERα reporter gene assay to measure the estrogenic activity of wastewater extracts as EEQ and the antiestrogenic activity as 4-hydroxy-tamoxifen equivalent quantity (TMXEQ). All samples were simultaneously analyzed for the expression of *vitellogenin-1* (*vtg1*) and *choriogenin-H* (*chgH*) in male medaka (*Oryzias latipes*) by quantitative real-time PCR (qPCR). In addition, estrone (E1), 17β-estradiol (E2), estriol (E3), and 17α-ethynylestradiol (EE2) were simultaneously analyzed by ultraperformance liquid chromatography coupled with tandem mass spectrometry (UPLC/MS/MS), and the concentrations were used to predict estrogenic activity (predicted EEQ). By comparing these indicators, we investigated whether the measured EEQ based on the in vitro ERα reporter gene assay is a reliable predictor of the induction of in vivo *vtg1*/*chgH* expression even when there is a large discrepancy between measured and predicted EEQs. Most recent studies of estrogenic activity in wastewater that used the in vitro ERα reporter gene assay used only a mammalian receptor-based assay.[16] However, our recent study revealed that the measured activity differed between human (*Homo sapiens*) and medaka ERαs.[9] So by comparing the measured EEQs, we also investigated whether the medaka ERα assay can predict the induction of in vivo *vtg1*/*chgH* expression in male medaka better than the human ERα assay.

Two studies have investigated changes in estrogenic and antiestrogenic activities during advanced treatment of secondary effluent (SE), and reported that estrogenic activity of wastewater measured by the yeast estrogen screen method increased after soil aquifer treatment because of the efficient removal of antiestrogenic activity.[11,17] The most important question is whether the in vivo estrogenic effect actually increases in this situation. To use the in vitro ERα reporter gene assay as a reliable predictor of in vivo estrogenic effects, this question must be resolved. To investigate this question, we also measured the changes in all indicators described above during wastewater reclamation processes. SE was treated by ultrafiltration (UF) membrane filtration either alone or followed by reverse osmosis (RO) membrane filtration. Because UF can remove estrogenic compounds partially[18,19] and RO can remove them completely,[8,18] we expected serial changes in all indicators during these reclamation processes.

MATERIALS AND METHODS

Chemicals. The chemicals used in this study are described in Supporting Information (SI) Methods S1.

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Wastewater Reclamation Process and Sampling. Wastewater reclamation experiments were carried out at a pilot plant within WWTP A in Japan. After conventional activated sludge treatment, secondary effluent (SE) released from the final settling tank received one of three wastewater reclamation processes: (1) coagulation followed by UF membrane filtration (SI Figure S1, Coag. + UF); (2) UF alone (UF); and (3) UF followed by RO (UF + RO).

Experiments were carried out six times (exp 1−6) from 2011 to 2013. SE and reclaimed water were collected in each experiment; in total, 20 samples were collected (Table 1). All samples for the reporter gene assay and chemical analysis were collected in 1-L amber glass bottles to which 1 g/L ascorbic acid was added as a preservative. The samples were transported at <16 °C to our laboratory at Kyoto University, where they were filtered and extracted within 48 h. At the same time, 20 L of each sample was collected and transported at <16 °C to the Public Works Research Institute, Tsukuba, Ibaraki, where male medaka were exposed to these water samples and qPCR analysis of in vivo *vtg1*/*chgH* expression was carried out (see Quantification of Hepatic vtg1 and chgH mRNA in Male Medaka by qPCR section).

Sample Treatment for In Vitro ERα Reporter Gene Assay and Chemical Analysis. Samples for the assay were extracted by solid-phase extraction (SPE) as described previously.[9] In brief, each 1-L sample was passed through a glass fiber filter (pore size 1 μm; GF/B, Whatman, Maidstone, UK). The filtrate was passed through a preconditioned SPE cartridge (Oasis HLB, 200 mg/6 cc, 30-μm particle size, Waters Corp., Milford, MA). The cartridge was dried for 2 h under gentle air pressure in a glass manifold. Then compounds trapped on the cartridge were eluted with 10 mL of methanol. The eluate was evaporated to dryness under a gentle nitrogen stream at 37 °C. The residue was immediately dissolved in 1 mL of cell culture medium (described below) containing 0.2% DMSO, followed by serial dilutions with the cell culture medium, and then used in the assay. The concentrations of wastewater extracts during cell exposure were defined as the relative enrichment factor (REF): the ratio of the enrichment factor (from the SPE step) to the dilution factor of the wastewater extracts in the ERα reporter gene assay. All samples were stored at −30 °C until the assay. Milli-Q water (Millipore Corp., Billerica, MA) was treated in parallel as a blank control, which we confirmed had no estrogenic or antiestrogenic activity by human and medaka ERα reporter gene assays.

We had already confirmed that the recovery rates of E1, E2, E3, and EE2 in wastewater during the SPE procedure were >85%.[9] So the estrogenic activity measured by the assay was directly comparable to in vivo *vtg1*/*chgH* levels determined by qPCR and to concentrations of estrogens determined by chemical analysis.

Chemical Analysis of Natural and Synthetic Estrogens. Concentrations of E1, E2, E3, and EE2 in SE or reclaimed water were measured by UPLC/MS/MS as described previously.[9,20] In brief, after filtration of wastewater samples through a GF/B filter, stable isotope surrogates (SI Methods S1) were added to the filtrate as internal standards. Wastewater samples were then extracted by SPE as described above with the addition of a Sep-Pak Plus NH2 cartridge (360 mg, aminopropyl, 55−105-μm partical size, Waters) below the dried Oasis HLB to reduce the effect of the sample matrix on ionization.

Evaluation of Estrogenic and Antiestrogenic Activities by In Vitro Assay. The ERα reporter gene assay was performed as described previously.[9] In brief, HEK 293 cells were seeded in 96-well plates (Nunc, Rochester, NY) in phenol-red−free Dulbecco's modified Eagle's medium (Sigma-Aldrich Co.) supplemented with 10% charcoal/dextran-treated fetal bovine serum (Hyclone, South Logan, UT). After 16 h incubation, cells were transfected with a reporter plasmid (to express firefly luciferase), ERα-expressing plasmid (to express human or medaka ERα), and an internal control plasmid (to express sea pansy luciferase) using Fugene 6 transfection reagent (Promega, Madison, WI) according to the manufacturer's instructions. After 4 h incubation, the diluted wastewater extracts were added to the medium. To avoid cell toxicity, the concentration of DMSO in serial dilutions never exceeded 0.1%. After 40 h incubation, luciferase activity was measured by a chemiluminescence assay with the Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured by an Infinite 200 multifunctional microplate reader (Tecan, Salzburg, Austria). The strength of transactivation was calculated as the ratio of firefly luciferase activity (from the reporter plasmid) to sea pansy luciferase activity (from the internal control plasmid), and expressed as a multiple of the vehicle control activity (cell culture medium including ≤0.1% DMSO). From the dose−response curves of estrogenic activity of wastewater extracts, the measured EEQ of wastewater extracts was calculated as described previously[9] (see SI Methods S2 for brief description).

Antiestrogenic activity was also evaluated as described previously.[9] In brief, 4-hydroxy-tamoxifen (4-OHT) was used as a standard E2 antagonist. Antiestrogenic activity detected by the assay was determined as the 4-OHT equivalent (TMXEQ). After plasmid transfection, serial dilutions of wastewater extracts were added to the test wells. Transfected cells were then stimulated with 5.0×10^{-11} M E2 (human ERα assay) or 5×10^{-9} M E2 (medaka ERα assay) to induce the maximum response of ER. After 40 h incubation, luciferase activity was measured. Other procedures were the same as for the evaluation of estrogenic activity. From the reduction of E2 activity, TMXEQ of wastewater extracts was calculated as described previously[9] (see SI Methods S2 for brief description).

For each assay, the activity of E2 (10^{-14} to 10^{-5} M) was analyzed in parallel as a positive control. All assays were performed at least twice, using duplicate sample points in each.

The cytotoxicity of each SE and reclaimed water extract was analyzed under the same experimental conditions as those for estrogenic/antiestrogenic activities by using a cell counting kit (CCK-8; Dojindo Molecular Technologies, Japan) as described previously.[9] In brief, the number of living cells was estimated by measuring the amount of formazan dye generated by the activity of dehydrogenases in cells, which is directly proportional to the number of living cells. Data obtained from the dilution range of extracts in which the number of living cells was significantly lower ($P < 0.05$) than in vehicle exposure cells were excluded from further analysis.

Data Presentation for In Vitro Assay. Measured EEQs, predicted EEQs, and measured TMXEQs were calculated as described previously[9] (see SI Methods S2 for brief description).

Quantification of Hepatic vtg1 and chgH mRNA in Male Medaka by qPCR. Male medaka (*Oryzias latipes*, strain d-rR) were exposed to samples, and relative expression levels of *vtg1* and *chgH* in the liver were measured by qPCR. Six male medaka 4−7 months old were exposed to SE, reclaimed water,

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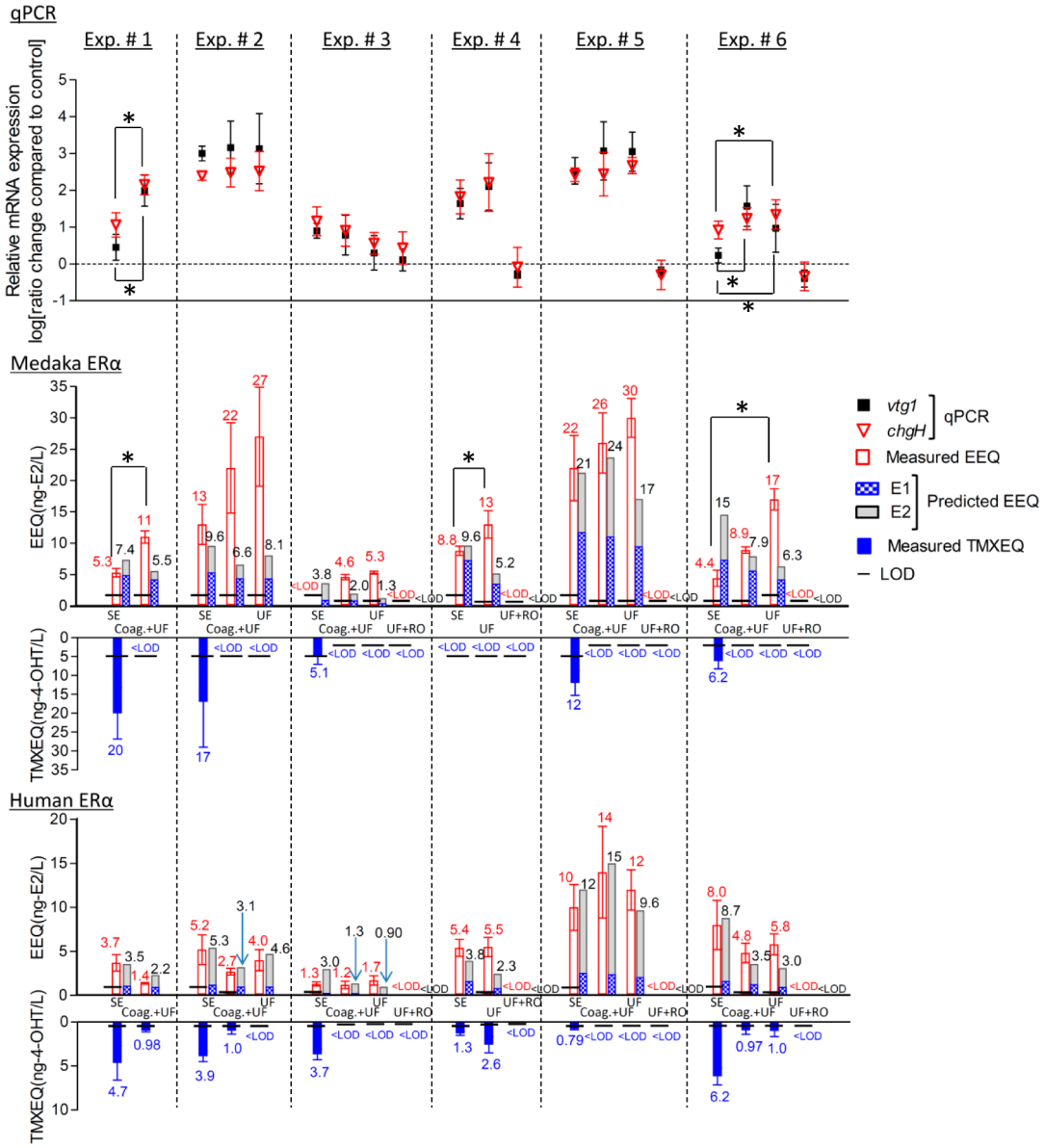

Figure 1. Comparison of predicted EEQ, measured EEQ, and measured TMXEQ, and *vtg1*/*chgH* levels in male medaka. (Top) In vivo *vtg1*/*chgH* levels in medaka exposed to wastewater samples, expressed as the ratio change relative to controls. Values are means ± SEM (n = 6). (Middle and bottom) Predicted EEQ, measured EEQ, and measured TMXEQ based on medaka ERα or human ERα. Values are means ± SEM (n = 4−8) for measured EEQ and TMXEQ. Horizontal bars indicate LOD. In some samples, LODs for human ERα are too small to indicate (<0.085 ng-E2/L for measured EEQ). SE: secondary effluent; coag + UF: coagulation followed by ultrafiltration; UF: UF alone; UF + RO: UF followed by reverse osmosis membrane filtration. *The gene expression levels or measured EEQs of reclaimed water are significantly higher than those of SE (p < 0.05).

or dechlorinated tap water (control) in a 3-L glass tank for 96 h under a 16/8-h light/dark cycle at 24 ± 1 °C. All water samples were replaced daily with water of the same origin. During the exposure period, the fish were not fed. After exposure, the fish were anesthetized with ice and the liver was removed from each (n = 6) and stabilized in RNAlater RNA stabilization solution (Life Technologies Corp., Carlsbad, CA). Total RNA was extracted by using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. The integrity and purity of the RNA were assessed with a Bioanalyzer (Agilent, Palo Alto, CA) according to the manufacturer's instructions. The average of RIN numbers for all samples was 7.2. RNA isolated from each of the six biological replicates was analyzed by qPCR (see below).

Exposure tests using E1 and E2 were conducted as positive control experiments. Male medaka were exposed to dechlorinated tap water containing E1 (nominal concentrations 2.0, 20, and 200 ng/L; 6 fish) or E2 (2.7, 27, and 270 ng/L; 10 fish) in an 8-L glass tank. The water was replaced daily. Other conditions were the same as for the exposure to wastewater. The concentrations of E2 as measured by chemical analysis were 2.9, 21, and 210 ng/L at 0 h, and 2.1, 18, and 160 ng/L after 24 h. After 96 h, the liver was sampled (n = 6 for E1, n = 10 for E2) and RNA was extracted as above.

First-strand cDNA was synthesized by reverse transcription using a QuantiTect Reverse Transcription Kit (Qiagen) with a blend of oligo-dT and random primers. A 2.5-μL aliquot of the 25-μL first-strand cDNA solution was analyzed by qPCR using a QuantiTect Probe PCR Kit (Qiagen). The *β-actin* gene was

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used as an internal control. Primers and probes used for medaka *vtg1*, *chgH*, and *β-actin* are described in SI Methods S3. These mRNAs were amplified and detected with a SmartCycler I system (Cepheid, Sunnyvale, CA) under thermal cycling conditions of 15 min at 95 °C, and 45 cycles of 1 s at 94 °C and 60 s at 60 °C.

Amounts of *vtg1*, *chgH*, and *β-actin* in livers were quantified by comparison with a reference standard curve derived from a medaka liver in which high concentrations of *vtg1* and *chgH* were induced by E2 exposure. First, the relative abundance of *vtg1* and *chgH* in test fish was normalized to *β-actin*. Then, the ratio change of the relative levels in test fish was calibrated against that in the control fish. The induction of *vtg1* or *chgH* expression by SE or reclaimed wastewater was expressed as a multiple of the induction by tap water exposure. Similarly, the ratio change of the relative levels in E1- or E2-treated fish were normalized to the controls. The ratio change of the mean *vtg1* and *chgH* expression levels was compared with the measured EEQ based on the in vitro ERα reporter gene assay and the predicted EEQ based on measured concentrations of E1, E2, E3, and EE2.

Statistical Analysis. Statistical differences ($p < 0.05$) in the results of the qPCR analysis and measured EEQ values were assessed by Student's *t*-test (GraphPad Prism 5; GraphPad Software, Inc., La Jolla, CA). The relationship between in vivo *vtg1*/*chgH* expression and measured or predicted EEQs, and between measured and predicted EEQs, was described by linear regression, and the significance of correlations was assessed by *F*-test (GraphPad Prism 5).

RESULTS AND DISCUSSION

Concentration−Response Curve of Estrogenic Activity and Concentration−Inhibition Curve of Antiestrogenic Activity. E1 and E2 were detected in all SE extracts (Table 1), but E3 was not detected. EE2 also was not detected in any samples, as the contraceptive pill is not a popular method of contraception in Japan.[21] In some experiments, E1 and E2 were reduced by coagulation + UF (exp 1−3, 6) and UF alone (exp 2−6). Reduction of natural estrogens by UF has been reported previously,[18,19] and fouled membrane could eliminate EDCs by adsorption and size exclusion.[19] After RO, E1 and E2 were not detected (exp 3−6), probably because of the tight size exclusion, in agreement with previous observations.[8,18] From the concentrations of E1 and E2, the predicted EEQs were calculated for medaka ERα and human ERα (SI Methods S2; Table 1).

Concentration−response curves of estrogenic activity and concentration−inhibition curves of antiestrogenic activity were obtained from the results of ERα reporter gene assays (SI Figure S2). Neither cytotoxicity of samples nor nonreceptor-mediated pathways such as adsorption of the agonist by large dissolved organic matter in the wastewater extracts was responsible for the decrease of the estrogenic response (SI Figure S2 "Cytotoxicity"; Discussion S1). The REF that gave 25% of the maximum activity of E2 ($EC_{25(extract)}$) was determined from the concentration−response curves (red), and the REF that gave a 25% reduction of activity of E2 ($IC_{25(extract)}$) was determined from the concentration−inhibition curves (blue). From the $EC_{25(extract)}$ and $IC_{25(extract)}$ values, we calculated the measured EEQ and TMXEQ values, respectively (SI Methods S2; Table 1; Figure 1).

Comparisons among Measured EEQ, Predicted EEQ, Measured TMXEQ, and In Vivo vtg1/chgH Levels. We confirmed that the in vivo medaka exposure and qPCR analyses could quantitatively detect the *vtg1* and *chgH* mRNAs induced in a dose dependent manner by E1 and E2 (SI Figure S3). We also confirmed that E1 and E2 were not detected by chemical analysis, and that estrogenic activity was not detected by the in vitro medaka ERα reporter gene assay, in the dechlorinated tap water used as a control for the medaka exposure experiment (data not shown).

Measured EEQs, predicted EEQs, measured TMXEQs, and in vivo *vtg1*/*chgH* levels are compared in Figure 1. By medaka ERα assay, all SE extracts except in expt 4 showed antiestrogenic activity (Medaka ERα, TMXEQ), and measured EEQs were lower than predicted (expt 1, 3, and 6). Interestingly, measured EEQ increased after coagulation + UF and UF alone in spite of the decrease of predicted EEQ, along with the removal of antiestrogenic activity in SE (expt 1−6). For example, in expt 6, after UF, predicted EEQ decreased from 15 to 6.4 but measured EEQ increased from 4.4 to 17. Importantly, in vivo *vtg1*/*chgH* levels also tended to increase after coagulation + UF and UF alone in these experiments (qPCR, expt 1, 4−6). For example, after UF in expt 6, $\log_{10}$ *vtg1* and *chgH* expression increased from 0.23 to 0.97 and from 0.92 to 1.3, respectively. Overall, it seems that measured EEQs by medaka ERα assay changed similarly to in vivo *vtg1*/*chgH* levels (see also SI Figure S4), but predicted EEQs based on chemical analysis did not (see also SI Figure S5). After RO, predicted EEQ, measured EEQ, measured TMXEQ, and in vivo *vtg1*/*chgH* inductions were not detected (Figure 1, expt 3−6, UF + RO).

By human ERα, all SE extracts showed both estrogenic and antiestrogenic activities (Figure 1, Human ERα, TMXEQ). After coagulation + UF or UF alone in most experiments, in contrast to medaka ERα, measured EEQ based on human ERα changed differently from in vivo *vtg1*/*chgH* levels (expt 1, 2, 4, 6). For example, after coagulation + UF in expt 1, measured EEQ decreased from 3.7 to 1.4, but *vtg1* levels increased from 0.45 to 2.0 and *chgH* levels increased from 1.1 to 2.2. Expt 6 showed a similar result. In expt 2, measured EEQ decreased after coagulation + UF or UF alone, but in vivo *vtg1*/*chgH* levels did not. In expt 4, measured EEQ did not change, but in vivo *vtg1*/*chgH* levels increased (see also SI Figure S4). After RO, measured EEQs were not detected (Figure 1, expt 3−6, RO).

Correlations among vtg1/chgH Levels, Measured EEQ, and Predicted EEQ. Using the data in Figure 1, we analyzed the correlations of in vivo *vtg1*/*chgH* levels with predicted EEQ and measured EEQ (Figure 2). Measured EEQ based on medaka ERα showed good correlations with in vivo *vtg1*/*chgH* levels (A: *vtg1*, $R^2 = 0.66$, $p < 0.0001$; *chgH*, $R^2 = 0.67$, $p < 0.0001$), stronger than correlations of predicted EEQ with in vivo *vtg1*/*chgH* levels (B: *vtg1*, $R^2 = 0.29$, $p < 0.05$; *chgH*, $R^2 = 0.37$, $p < 0.05$). On the other hand, measured EEQ based on human ERα showed very weak correlations with *vtg1*/*chgH* levels (C: *vtg1*, $R^2 = 0.18$, $p > 0.05$; *chgH*, $R^2 = 0.23$, $p > 0.05$). Predicted EEQ based on human ERα also showed very weak correlations (D: *vtg1*, $R^2 = 0.26$, $p < 0.05$; *chgH*, $R^2 = 0.31$, $p < 0.05$). These results clearly show that measured EEQ based on medaka ERα is a better predictor of the induction of *vtg1*/*chgH* in male medaka than predicted EEQ by medaka and human ERα (based on the concentrations of E1 and E2) and measured EEQ based on human ERα.

Taking all results of Figures 1, 2, and SI S4 and S5 together, we conclude that measured EEQ based on the medaka ERα

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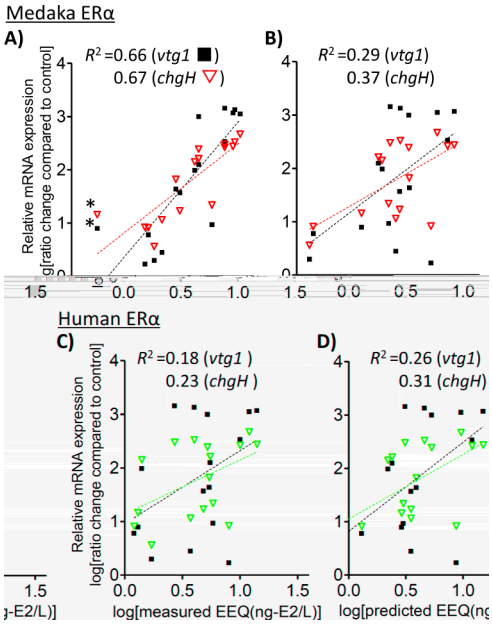

Figure 2. Correlations of mean hepatic *vtg1/chgH* levels in male medaka with (A and C) measured EEQs and (B and D) predicted EEQs based on medaka or human ERα. Post-RO data are excluded because *vtg1/chgH* were not induced (i.e., relative mRNA expression values were near 0), and measured EEQs were lower than the limit of detection (LOD). *Estrogenic activity was not detected but *vtg1/chgH* were induced in this sample (expt 3 SE), and therefore LODs of measured EEQ are plotted. Dashed lines show linear regressions.

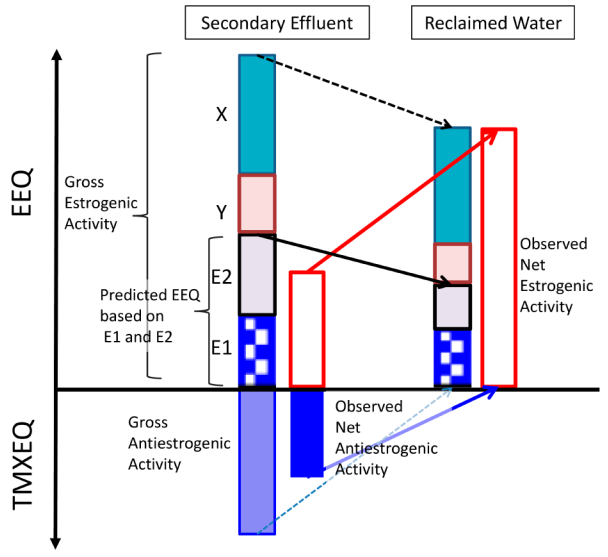

Figure 3. Model of changes in measured EEQ based on medaka ERα reporter gene assay and in vivo *vtg1/chgH* induction during ultrafiltration with or without coagulation. X and Y represent other estrogenic compounds besides natural estrogens.

assay is the best indicator of in vivo *vtg1/chgH* expression in 96 h static exposure to wastewater. The ability of measured EEQ based on in vitro assay to predict in vivo estrogenic activity in long-term exposure remains to be investigated (SI Discussion S2).

Measured and predicted EEQs of each sample based on the data in Figure 1 were directly compared (SI Figure S6). By medaka ERα, several higher values of measured EEQ than predicted indicate the presence of other estrogenic compounds besides E1, E2, E3, and EE2. The human ERα assay showed good correlation between predicted EEQ and measured EEQ, which indicated a smaller contribution of these to measured EEQ. Bisphenol A and alkylphenols such as 4-nonylphenol are possible candidates for medaka ERα-specific estrogenic activity in wastewater (SI Discussion S3).

Importance of Species-Appropriate In Vitro Assay for Predicting In Vivo Estrogenic Effects. One study compared fish and mammalian ER properties in the reporter gene assay and concluded that ER transactivation in one vertebrate species could be extrapolated to another species for chemical screening.[22] Other studies, however, reported that sensitivities of ERα to estrogenic compounds differed between human and fish ERα,[23,24] and that rainbow trout ERα was more accurate than human ERα at predicting the in vivo estrogenic activity of compounds in rainbow trout.[23] Our results also indicate the importance of species-appropriate in vitro assays for predicting in vivo estrogenic effects.

Model of Changes in Measured EEQ Based on ERα Reporter Gene Assay and In Vivo vtg1/chgH Induction during Wastewater Reclamation Processes. After coagulation + UF and UF alone, measured EEQs based on medaka ERα and in vivo *vtg1* levels increased in spite of the decrease of predicted EEQ (Figures 1 and SI S4). The model in Figure 3 is one model that explains this phenomenon. In this model (1) estrogenic activity is suppressed by antiestrogenic activity, as demonstrated in our previous study;[9] (2) other estrogenic compounds besides E1, E2, E3, and EE2 (represented by X and Y, see SI Discussion S3) occur in SE and reclaimed water; and (3) antiestrogenic activity is removed more efficiently than estrogenic activity during wastewater reclamation process. In the model, the "gross" estrogenic activity is higher than predicted EEQ based on the concentrations of E1 and E2. In the balance between gross estrogenic and antiestrogenic activities, the observed net estrogenic activity is suppressed to below the gross activity, or even below the predicted EEQ. During wastewater reclamation processes, antiestrogenic activity (blue dashed arrow) is removed more efficiently than estrogenic activity (black dashed arrow). Therefore, the balance between gross estrogenic and antiestrogenic activities changes, and the observed net estrogenic activity could become higher than that before the reclamation process (red arrow), even though the predicted EEQ based on the concentrations of E1 and E2 is partially reduced (black arrow). This model can explain the change in measured EEQs based on medaka ERα and in vivo *vtg1* level (Figure 1, expt 1, 2, 4–6, coag + UF, and UF). The change in measured EEQs based on human ERα can be explained with another model (SI Figure S7), in which E1 and E2 explain most of the gross estrogenic activity, and the efficiencies of the removal of gross estrogenic and antiestrogenic activities during wastewater reclamation processes are similar.

Several studies have investigated changes in estrogenic and antiestrogenic activities during advanced treatment of SE.[8,11,17,25−27] Estrogenic activity of wastewater measured by the yeast estrogen screening method increased after soil aquifer treatment because of the efficient removal of antiestrogenic activity.[11,17] On the other hand, antiestrogenic activity measured by the same method increased after ozonation, possibly because of the efficient removal of estrogenic activity during ozonation.[26] These studies and our results demonstrate that net estrogenic activity measured by in vitro assay and in vivo *vtg1/chgH* expression could even increase during advanced

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treatment of SE, depending on the balance between gross estrogenic and antiestrogenic activities. These results indicate that to understand the change of estrogenic activity during advanced treatment of SE by in vitro assay, measurement of both estrogenic and antiestrogenic activities is essential.

In Vivo Mixture Effect of Estrogenic and Antiestrogenic Activities in Wastewater. Our results demonstrate that estrogenic and antiestrogenic activities co-occur in wastewater, and that in vivo *vtg1/chgH* expression in male medaka in 96 h static exposure to wastewater was determined by the balance of estrogenic and antiestrogenic activities. However, it remains unclear how other end points besides in vivo *vtg1/chgH* expression, such as reproductive performance (e.g., fecundity and fertility), are affected by exposure to wastewater which contains both estrogenic and antiestrogenic activities. In fact, Sun et al. reported that tamoxifen could neutralize the effect of E2 on plasma vitellogenin concentration in medaka, but fecundity and fertility were barely changed or even lessened.[28] Whereas considerable progress has been made in assessing the effects of mixtures of components with the same effect (e.g., estrogenic and antiandrogenic),[29−31] only a few studies have investigated the in vivo effects of mixtures of compounds with opposite effects, such as estrogenic and antiestrogenic.[28,32,33] Further studies of the in vivo effects of mixtures of estrogenic and antiestrogenic activities in the aquatic environment are required.

To the best of our knowledge, this is the first study to show quantitatively that (1) the net estrogenic activity measured by the in vitro ERα reporter gene assay can predict the induction of in vivo *vtg1/chgH* expression better than chemical analysis even when estrogenic and antiestrogenic activities coexist; (2) in vivo *vtg1/chgH* expression in male medaka in 96 h static exposure to wastewater was determined by the balance of estrogenic and antiestrogenic activities; and (3) the medaka ERα reporter gene assay can predict in vivo *vtg1/chgH* expression better than the human ERα reporter gene assay.

■ ASSOCIATED CONTENT

Supporting Information

Schematic diagram of the wastewater reclamation processes; dose−response curves of estrogenic and antiestrogenic activities of wastewater and reclaimed water; *vtg1* and *chgH* induction in medaka by E1 and E2 exposure; changes in measured EEQs by in vitro ERα reporter gene assay and in vivo *vtg1* levels during wastewater reclamation; changes in predicted EEQs based on medaka ERα and in vivo *vtg1* levels during wastewater reclamation; correlations between measured and predicted EEQs; model of changes in measured EEQ based on human ERα during wastewater reclamation process; methods for other experiments; discussion of the cytotoxicity of samples and nonreceptor-mediated pathways; the ability of measured EEQ based on in vitro assay to predict in vivo estrogenic effect; and possible candidate compounds for medaka ERα-specific estrogenic activity. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b01027.

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Notes

The authors declare no competing financial interest.

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