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# Screening of agonistic activities against four nuclear receptors in wastewater treatment plants in Japan using a yeast two-hybrid assay

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**Abstract**

To assess the potential endocrine disruptive effects through multiple nuclear receptors (NRs), especially non-steroidal NRs, in municipal wastewater, we examined the agonistic activities on four NRs (estrogen receptor α, thyroid hormone receptor α, retinoic acid receptor α and retinoid X receptor α) of untreated and treated wastewater from municipal wastewater treatment plants (WWTPs) in Japan using a yeast two-hybrid assay. Investigation of the influent and effluent of seven WWTPs revealed that agonistic activities against steroidal and non-steroidal NRs were always detected in the influents and partially remained in the effluents. Further investigation of four WWTPs employing conventional activated sludge, pseudo-anoxic-oxic, anoxic-oxic and anaerobic-anoxic-oxic processes revealed that the ability to reduce the agonistic activity against each of the four NRs varies depending on the treatment process. These results indicated that municipal wastewater in Japan commonly contains endocrine disrupting chemicals that exert agonistic activities on steroidal and non-steroidal NRs, and that some of these chemicals are released into the natural aquatic environment. Although the results obtained in yeast assays suggested that measured levels of non-steroidal NR agonists in the effluent of WWTPs were not likely to cause any biological effect, further study is required to assess their possible risks in detail.

**Key words**: agonistic activity; endocrine disrupting chemicals; non-steroidal nuclear receptor; wastewater treatment plants

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## Introduction

Endocrine disrupting chemicals (EDCs) are synthetic or natural compounds that have the potential to disrupt or alter functions of the endocrine system and consequently cause detrimental effects in intact organisms. EDCs primarily exert their endocrine disruptive effects through direct interaction with nuclear receptors (NRs). Earlier studies have focused on EDCs that interact with steroidal receptors such as estrogen receptor (ER) and androgen receptor (Campbell et al., 2006; Khanal et al., 2006). However, it has been shown that the NR superfamily contains many different receptors in eukaryotic organisms (Chawla et al., 2001), and it has been suggested that each of the NRs can serve as the active site for the endocrine disruptive effects of EDCs (Janošek et al., 2006; Tabb and Blumberg, 2006). As a result, not only steroidal NRs, but also non-steroidal NRs should be included in the assessment of potential endocrine disruptive effects in the environment.

Wastewater treatment plants (WWTPs) receive a large amount of municipal and industrial wastewater, which may contain various EDCs. A number of studies have been conducted to evaluate the occurrence and fate of estrogenic and androgenic EDCs in WWTPs (Sumpter, 1995; Kirk et al., 2002; Svenson et al., 2003; Leusch et al., 2006; Vajda et al., 2008). By contrast, little attention has been given to the occurrence of EDCs that interfere with non-steroidal NRs. Only recently, a few studies regarding the disruptive activity on thyroid hormone receptor (TR) (Ishihara et al., 2009; Jugan et al., 2009; Murata and Yamauchi, 2008) and retinoic acid receptor (RAR) (Schoff and Ankley, 2002; Zhen et al., 2009) in the influent and effluent of WWTPs have been published. To assess the potential endocrine disruptive effects of wastewater that occur through a variety of NRs, it is necessary to determine what type of NRs are interfered with by EDCs in wastewater, how effectively the biological wastewater treatment processes employed in WWTPs can remove such EDCs, and whether the contamination level in the WWTP effluent can cause biological effects in the receiving rivers.

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In this study, we examined the agonistic activities of wastewater samples from municipal WWTPs in Japan on ERα (a representative steroidal NR) and three non-steroidal NRs, TRα, RARα and retinoid X receptor (RXR) α, using a yeast two-hybrid assay (Nishikawa et al., 1999). First, the agonistic activities of influent and effluent samples from seven WWTPs against four NRs were measured. Second, different biological treatment processes were compared for their effectiveness at reducing agonistic activities of wastewater against the aforementioned NRs. With the exception of ER, very little is known about EDCs that interfere with target NRs in wastewater; therefore, we chose a bioassay capable of assessing the effects of all active known and unknown contaminants.

# 1 Materials and methods

## 1.1 Chemicals

17β-Estradiol (E2; ⩾ 98%), 3,3′,5-triiodo-L-thyronine (T3; ⩾ 95%), 3,3′,5-triiodothyroacetic acid (TRIAC; approx. 95%), all-*trans*-retinoic acid (atRA; ⩾ 98%), 9-*cis*-retinoic acid (9cRA; 98%) and methanol (MeOH; ⩾ 99.9% (HPLC grade)) were obtained from Sigma-Aldrich (St. Louis, USA). Dimethyl sulfoxide (DMSO; ⩾ 99.9%) was obtained from Wako Pure Chemical Industries (Osaka, Japan).

## 1.2 Wastewater samples

Grab wastewater samples were collected from seven WWTPs in Osaka Prefecture (WWTPs-A to -G) and three WWTPs in Toyama Prefecture (WWTPs-H to -J), Japan. WWTPs-B, -C, -E, -H, -I and -J, WWTP-A, WWTP-F, and WWTPs-D and -G applied conventional activated sludge (CAS), pseudo-anoxic-oxic (pAO), AO, and anaerobic-anoxic-oxic ($A_2O$) processes for biological treatment, respectively. All the investigated WWTPs receive mainly domestic wastewater and small amounts of industrial discharges. To screen for agonistic activities against NRs in wastewater, influent (after the primary settling tank) and effluent (after the final settling tank) samples were collected from WWTP-A in December 2006, WWTP-B in January 2007, WWTPs-C and -D in September 2006

**Table 1** Characteristics of influent and effluent samples

| WWTP* | Sampling date | Sample | Temp. (°C) | pH | DOC (mg/L) | TN (mg/L) |
|---|---|---|---|---|---|---|
| A (pAO) | December 4, 2006 | Influent | na | 8.4 | 63 | 27 |
| | | Effluent | na | 7.6 | 11 | na |
| | December 12, 2008 | Influent | 21.8 | 7.7 | 38 | 39 |
| | | Effluent | 22.5 | 6.7 | 11 | 18 |
| | December 16, 2008 | Influent | 21.1 | 7.3 | 51 | 40 |
| | | Effluent | 22.4 | 6.8 | 14 | 20 |
| | December 25, 2008 | Influent | 21.3 | 6.9 | 46 | 44 |
| | | Effluent | 21.7 | 6.8 | 12 | 22 |
| B (CAS) | January 11, 2007 | Influent | na | 8.0 | na | 31 |
| | | Effluent | na | 7.0 | na | 12 |
| C (CAS) | September 26, 2006 | Influent | na | 8.1 | 43 | 11 |
| | | Effluent | na | 7.6 | 5.6 | 8.3 |
| D ($A_2O$) | September 26, 2006 | Influent | na | 7.4 | 25 | 17 |
| | | Effluent | na | 7.2 | 5.3 | 3.8 |
| E (CAS) | December 12, 2008 | Influent | 19.9 | 7.7 | 25 | 36 |
| | | Effluent | 20.8 | 6.7 | 4.9 | 11 |
| | December 16, 2008 | Influent | 19.5 | na | 33 | 35 |
| | | Effluent | 20.2 | 6.7 | 6.8 | 9.4 |
| | December 25, 2008 | Influent | 18.8 | 7.6 | 26 | 31 |
| | | Effluent | na | 7.1 | 5.7 | 8.0 |
| F (AO) | December 12, 2008 | Influent | 20.4 | 7.3 | 25 | 26 |
| | | Effluent | 20.9 | 6.4 | 4.6 | 13 |
| | December 16, 2008 | Influent | 19.8 | 7.0 | 38 | 27 |
| | | Effluent | 20.5 | 6.4 | 8.6 | 11 |
| | December 25, 2008 | Influent | 19.5 | 7.4 | 28 | 28 |
| | | Effluent | 19.9 | 6.4 | 6.6 | 11 |
| G ($A_2O$) | December 12, 2008 | Influent | 20.1 | 7.3 | 20 | 38 |
| | | Effluent | 20.8 | 6.3 | 3.7 | 11 |
| | December 16, 2008 | Influent | 19.5 | 7.4 | 21 | 35 |
| | | Effluent | 20.3 | 6.5 | 5.9 | 9.8 |
| | December 25, 2008 | Influent | 19.5 | 7.7 | 20 | 41 |
| | | Effluent | 20.2 | 6.4 | 4.3 | 12 |
| H (CAS) | October 3, 2006 | Influent | na | 7.2 | 30 | 33 |
| | | Effluent | na | 6.8 | 4.4 | 10 |
| I (CAS) | October 3, 2006 | Influent | na | 8.5 | 23 | 37 |
| | | Effluent | na | 7.5 | 5.5 | 13 |
| J (CAS) | October 3, 2006 | Influent | na | 7.2 | 29 | 18 |
| | | Effluent | na | 7.0 | 2.7 | 14 |

DOC: dissolved organic carbon; TN: total nitrogen; AO: anoxic-oxic; $A_2O$: anaerobic-anoxic-oxic; CAS: conventional activated sludge; pAO: pseudo-anoxic-oxic.

* Biological treatment processes are shown in parentheses.

na: not analyzed.

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and WWTPs-H, -I and -J in October 2006. To assess the ability of each biological process to reduce the agonistic activities of wastewater against NRs, triplicate samples were collected after the primary and final settling tanks and each stage of biological treatment in WWTPs-A, -E, -F and -G in December 2008. All the samples were taken in the morning or early in the afternoon (between 10 a.m. and 2 p.m.). All samples were transported to the laboratory on ice and subjected to solid phase extraction (SPE) within 24 hr. The representative characteristics of samples after the primary and final settling tanks are listed in Table 1.

### 1.3 Sample preparation

Samples applied to the yeast two-hybrid assay were prepared as previously described (Inoue et al., 2009). Briefly, wastewater samples were filtered through a Whatman GF/B filter (Whatman, UK) to avoid clogging the SPE cartridge, and 2 L of the filtrates without pH adjustment were passed through Oasis HLB cartridges (6 mL/500 mg; Waters, USA) that had been conditioned with 6 mL of MeOH and ultra pure water at a flow rate of 5 to 10 mL/min. After sample loading, the cartridge was washed with 6 mL of ultra pure water and dried. Thereafter, the bound substances were eluted with 6 mL of MeOH and dried under a gentle nitrogen flow. The dried residues were then dissolved in 200 μL of DMSO, which resulted in a concentration factor of 10,000 when compared with the original wastewater samples. The concentrated samples were stored in the dark at 4°C until analysis. Prior to the bioassays, the samples were serially diluted with DMSO.

### 1.4 Yeast two-hybrid assay

The agonistic activities of the wastewater samples against the NRs were measured by a yeast two-hybrid assay employing the recombinant yeast, *Saccharomyces cerevisiae* Y190, which contained human NR (ERα, TRα, RARα or RXRα) and the coactivator TIF2 (Nishikawa et al., 1999). Assays were conducted as previously described (Inoue et al., 2009). The final concentration factors of the test samples applied to the assay system ranged from 0.1 to 100. A negative control experiment without the test sample (1% DMSO) and positive control experiments with varying concentrations of E2, T3 or TRIAC, atRA and 9cRA dissolved in DMSO for ERα, TRα, RARα and RXRα, respectively, were also performed. E2, T3, TRIAC, atRA and 9cRA were added to the assay system at $1.0 \times 10^{-11}$ to $5.0 \times 10^{-6}$ mol/L, $1.0 \times 10^{-10}$ to $1.0 \times 10^{-4}$ mol/L, $1.0 \times 10^{-11}$ to $5.0 \times 10^{-6}$ mol/L, $1.0 \times 10^{-11}$ to $5.0 \times 10^{-6}$ mol/L and $1.0 \times 10^{-11}$ to $1.0 \times 10^{-4}$ mol/L, respectively, to obtain the dose-response curves. All assays were conducted in triplicates unless otherwise noted. The relative agonistic activity (%) of the test sample was calculated by setting the maximum β-galactosidase activity of the positive sample to 100% and the activity of the negative control to 0%. When the relative agonistic activity of a test sample against an NR exceeded 10%, the concentration equivalent to the natural ligand of the NR was calculated using the following equation, according to the method developed to evaluate the estrogenicity of environmental samples (Kawagoshi et al., 2003): equivalent concentration (ng/L) = (the concentration of natural ligand (ng/L) that gave 10% relative NR agonistic activity)/(the concentration factor of the test sample that gave 10% relative NR agonistic activity).

### 1.5 Statistical analysis

Data obtained from the yeast two-hybrid assay were analyzed using SPSS software ver. 15.0 for Windows (SPSS Inc., USA). A student's *t*-test was used to compare the NR agonistic activity of wastewater samples with that of the negative control. Differences were considered significant and highly significant at $p < 0.05$ and $p < 0.01$, respectively.

## 2 Results and discussion

### 2.1 NR agonistic activities of wastewater samples

To elucidate the occurrence of EDCs interfering with multiple NRs, we examined the agonistic activities against ERα, TRα, RARα and RXRα in influent and effluent samples collected from seven WWTPs between September 2006 and January 2007. The results are presented in Fig. 1.

#### 2.1.1 ERα agonistic activity in wastewater

All influent samples from seven WWTPs showed a highly significant ERα agonistic activity at a concentration factor of 100 (Fig. 1a). Highly significant ERα agonistic activity was also detected in effluent samples from WWTPs-A, -B, -C and -I at concentration factors of 10 and 100. Effluent samples from WWTPs-B and -C showed highly significant activities, even at the original wastewater concentration (concentration factor of 1). The maximum activity of the effluent sample was observed in WWTP-B (the relative ERα agonistic activities at concentration factors of 10 and 100 were 33.9% and 32.6%, respectively), and the E2 equivalent concentration of that sample was estimated to be 31 ng E2/L. In previous studies, the E2 equivalent levels of WWTP effluents determined using a yeast assay ranged from 4 to 35 ng/L in Japan (Onda et al., 2002), 35 to 147 ng/L in the United States (Tilton et al., 2002), 0.2 to 7.7 ng/L in Switzerland (Vermeirssen et al., 2006), <0.1 to 15 ng/L in Sweden (Svenson et al., 2003), undetectable to 40 ng/L in the United Kingdom (Kirk et al., 2002), 34 to 66 ng/L in Germany (Pawlowski et al., 2004) and undetectable to 42 ng/L in Australia (Mispagel et al., 2005). Thus, the contamination levels of our samples appeared to be comparable to previously reported contamination levels.

#### 2.1.2 TRα agonistic activity in wastewater

Highly significant agonistic activity against TRα was detected in all influent samples at concentration factors of 10 and/or 100 (Fig. 1b). Effluent samples from WWTPs-A, -B, -C, -D and -H had highly significant TRα agonistic activity, while those from WWTPs-I and -J had significant activity. The effluent sample from WWTP-D showed the highest relative TRα agonistic activity of 11.7% at a concentration factor of 100. The estimated T3 equivalent

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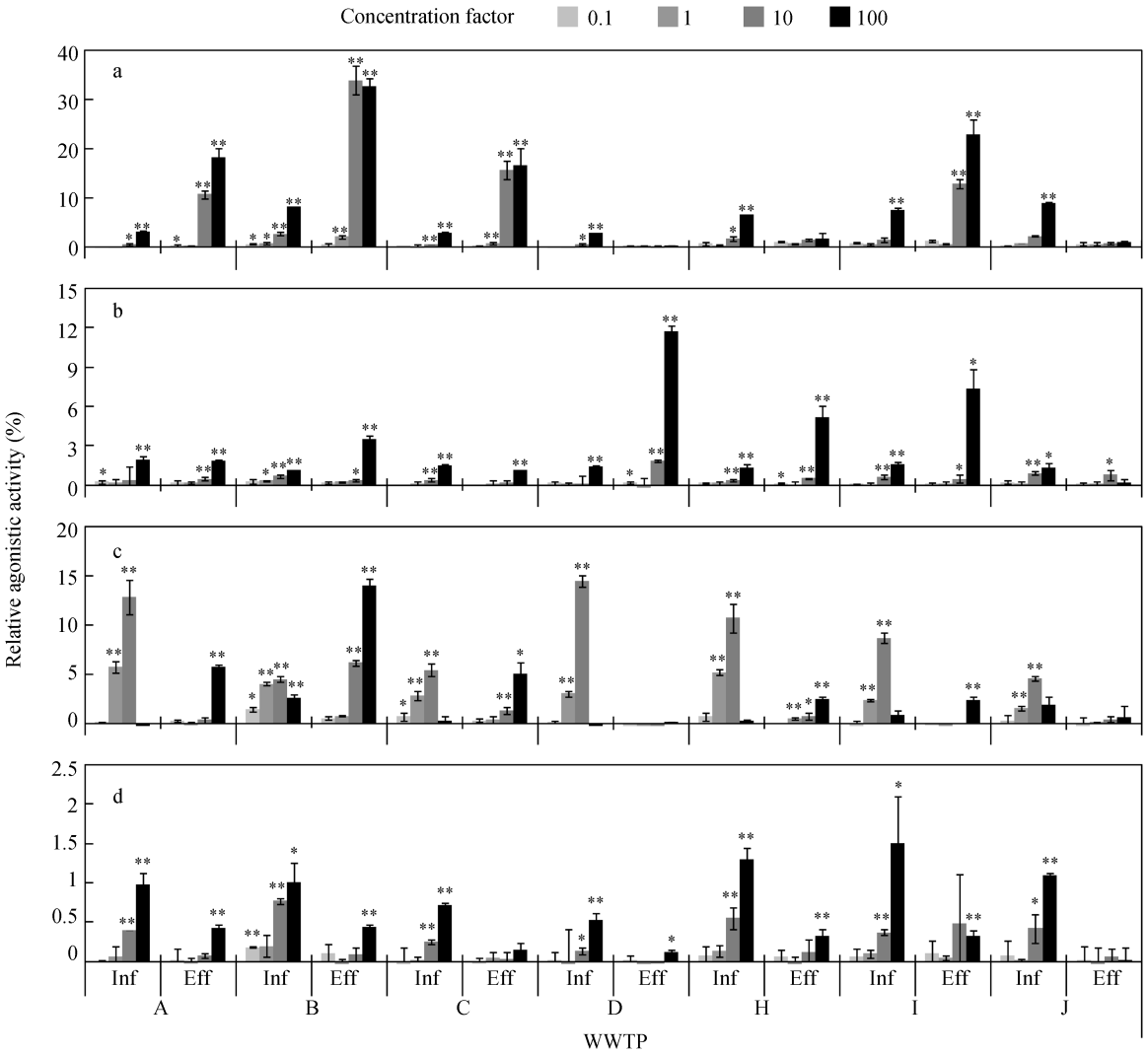


**Fig. 1** Relative agonistic activity of influent (Inf) and effluent (Eff) samples from four WWTPs in Osaka (WWTPs-A, -B, -C and -D) and three WWTPs in Toyama (WWTPs-H, -I and -J) on ERα (a), TRα (b), RARα (c) and RXRα (d) as determined by a yeast two-hybrid assay. Values shown are the means ± SD ($n$ = 3). The error bars for the TRα agonistic activity of effluent from WWTP-G at a concentration factor of 100, RXRα agonistic activity of influent from WWTP-A at a concentration factor of 10 show the data range of duplicate analyses. *$p < 0.05$ (significant activity), **$p < 0.01$ (highly significant activity) versus the negative control based on a Student's $t$-test.

concentration of the effluent sample from WWTP-D was 43 ng T3/L, which is higher than that detected in WWTPs in Paris, France (⩽1.3 ng T3/L) (Jugan et al., 2009).

### 2.1.3 RARα agonistic activity in wastewater

The RARα agonistic activity of influent samples was highly significant at a concentration factor of 1 in all WWTPs (Fig. 1c). However, the RARα agonistic activities at a concentration factor of 100 were lower than those at concentration factors of 1 and 10. Because detrimental effects on yeast growth were not observed at concentration factors as high as 100 (data not shown), the reduction of RARα agonistic activities at that concentration was likely due to the coexistence of interfering compounds that mask the RARα agonistic activity in our samples. Although no significant activity was detected in effluent samples from WWTPs-D and -J, effluent samples from WWTPs-A, -B, -C, -H and -I were all found to have highly significant RARα agonistic activity. The effluent sample from WWTP-B had the highest relative RARα agonistic activity of 14.0% at a concentration factor of 100, and its atRA equivalent concentration was estimated to be 17 ng atRA/L. This contamination level was slightly higher than the contamination level in the effluent of WWTPs in Beijing, China, where the highest atRA equivalent level in WWTP effluent was 3.2 ng atRA/L (Zhen et al., 2009).

### 2.1.4 RXRα agonistic activity in wastewater

All influent samples exhibited a highly significant RXRα agonistic activity (Fig. 1d). Effluent samples from WWTPs-A, -B, -H and -I also showed highly significant activity, while those from WWTP-D were found to have significant activity. However, the highest relative RXRα agonistic activity of the effluent sample was 0.43% (WWTP-B). Due to the very low agonistic activity, the 9cRA equivalent could not be calculated.

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#### 2.1.5 Preliminary risk assessment

As mentioned above, RXRα agonistic activity in WWTP effluents was very low and 9cRA equivalent could not be calculated. Thus, the detrimental effects of RXR agonists in WWTP effluents would be ignorable. Therefore, the possibility of biological effects on aquatic animals by ER, TR and RAR agonists in WWTP effluents was assessed by comparing the contamination level in WWTP effluent estimated in this study and the natural ligand concentration at which detrimental effect was observed in previous studies.

Vitellogenin is a commonly used *in vivo* biomarker for estrogenicity. Van den Belt et al. (2003) reported that significant vitellogenin induction occurred in juvenile rainbow trout exposed to 20 ng/L of E2 for 3 weeks. Holbech et al. (2006) also showed that vitellogenin is significantly induced in juvenile zebrafish by the exposure to 54 ng/L of E2 from 20 to 34 days post hatch. The ERα agonist contamination level detected in this study (maximal E2 equivalent concentration: 31 ng E2/L) was similar to these E2 level that induced vitellogenin in juvenile fish, suggesting that direct exposure to WWTP effluent for a prolonged period may cause some ER-mediated biological effects.

It was reported that exogenous T3 at 3.3 μg/L caused increased pigmentation and accelerated hatching in zebrafish embryos (Walpita et al., 2007). In this study, maximal T3 equivalent concentration of WWTP effluent was estimated to be 43 ng T3/L. Thus, the contamination level in WWTP effluent was much lower than the toxic level of T3.

RAR ligands are well known as potential teratogens in developing vertebrate embryos, and chronic exposure to atRA at concentrations equal to hundreds of nanograms a liter or more can cause a variety of dysmorphogenesis (Inoue et al., 2010). Degitz et al. (2003) reported that chronic exposure of atRA at ⩾600 ng/L for ⩾3 days caused a wide spectrum of malformations in African clawed frogs (*Xenopus laevis*) embryos. Herrmann (1995) also reported the occurrence of oedema and deformities of brain and tail in zebrafish embryos by the exposure of atRA at 900 ng/L from stage 13 to 23. Thus, the RARα agonist contamination level in WWTP effluent observed in this study (maximal atRA equivalent concentration: 17 ng atRA/L) were much lower than the ecotoxicological level.

Consequently, preliminary risk assessment suggested that the measured level of TRα, RARα and RXRα agonists in WWTP effluent were not likely to cause any biological effect. However, it has been reported that the E2 equivalent concentrations of nonylphenol and bisphenol A (the representative ER agonistic EDCs) required to exert the ER-mediated biological effects are lower than the concentration of E2 that induces adverse effects (Bevan et al., 2003; Sone et al., 2004). Therefore, the possibility of biological effects for TRα, RARα and RXRα agonists cannot be ruled out if the causative compounds are xenobiotic compounds, and identification of causative compounds is needed for detailed risk assessment.

### 2.2 Removal of agonistic activities against NRs in wastewater by different biological treatment processes

The ability of four different biological wastewater treatment processes (CAS, pAO, AO and $A_2O$) to reduce ERα, TRα, RARα and RXRα agonistic activities in wastewater were compared using samples from WWTPs-A (pAO), -E (CAS), -F (AO) and -G ($A_2O$). Due to the temporal variation in contaminant input and treatment efficiency in WWTPs, sampling was conducted on three distinct dates in December 2008. Significant agonistic activities against ERα, TRα, RARα and RXRα were detected in the influent samples from the four WWTPs at concentration factors of 50–100, 100, 10 and 100, respectively. The agonistic activities against the four NRs in municipal wastewater generally declined in response to the activated sludge treatments; however, the performance of each biological treatment process differed depending on the target NR. The ability of the four WWTPs to reduce the agonistic activities against NRs in wastewater is summarized in Table 2. Comparison of wastewater treatment performance (removal of DOC and TN) and removal of NRs agonistic activity revealed that removal of NRs agonistic activity had no significant correlation with wastewater treatment performance in the four investigated WWTPs (data not shown).

#### 2.2.1 Removal of ERα agonistic activity

In all four WWTPs, ERα agonistic activity after the first biological treatment tank increased by 1.5- to 4.0-fold when compared with that of the influents (after the primary settling tank) (Table 2). This may have resulted from the release of free estrogens by the cleavage of conjugated natural estrogens (Ternes et al., 1999) and/or the partial/complete removal of ER antagonists. ERα agonistic activity was further increased in the final settling tank in pAO. In CAS, although the activity declined in the final settling tank, the activity was still higher than the original activity in the influent. In AO and $A_2O$, ERα agonistic activity declined at the second and third biological treatment tanks. Additionally, the activity reached 0% at the final settling tank on 2 sampling dates in AO. Conversely, in $A_2O$, the activity increased to levels similar to or slightly higher than that of influent in the final settling tank. Overall, the ability to reduce ERα agonistic activity was AO > $A_2O$ ⩾ CAS > pAO. As suggested in a previous study (Hashimoto et al., 2007), the good performance in AO and $A_2O$ may have been due to their long sludge retention times (9.2–13.2 and 8.6–12.2 days in AO and $A_2O$, respectively) when compared with the sludge retention times in CAS and pAO (3.1–4.1 and 4.0–4.2 days in CAS and pAO, respectively).

#### 2.2.2 Removal of TRα agonistic activity

TRα agonistic activity declined in the first biological treatment tank in all WWTPs (Table 2). The decrease in activity was larger in CAS and pAO than that in AO and $A_2O$, and the activity reached 0% on 2 sampling dates in CAS. In AO, the TRα agonistic activity decreased further

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**Table 2** Ability of different biological treatment processes to reduce the agonistic activities of NRs

| WWTP | Tank | Remaining agonistic activity (%) | | | |
|---|---|---|---|---|---|
| | | ERα | TRα | RARα | RXRα |
| E (CAS) | Aeration tank | 403.4 ± 82.6 (0) | 3.4 ± 5.8 (2) | 7.7 ± 8.1 (1) | 8.7 ± 15.1 (2) |
| | Final settling tank | 144.4 ± 74.3 (0) | 4.3 ± 6.6 (1) | 9.5 ± 10.2 (1) | 8.1 ± 13.9 (2) |
| A (pAO) | Treatment tank | 146.5 ± 159.0 (0) | 30.0 ± 8.5 (0) | 66.2 ± 35.9 (0) | 40.2 ± 11.9 (0) |
| | Final settling tank | 383.6 ± 119.8 (0) | 36.3 ± 17.9 (0) | 36.4 ± 36.5 (0) | 33.3 ± 15.4 (0) |
| F (AO) | Anoxic tank | 318.2 ± 56.4 (0) | 46.1 ± 12.7 (0) | 95.2 ± 22.2 (0) | 24.0 ± 17.2 (0) |
| | Oxic tank | 147.4 ± 84.3 (0) | 14.6 ± 6.2 (0) | 5.2 ± 4.6 (1) | 11.6 ± 13.9 (0) |
| | Final settling tank | 37.1 ± 64.3 (2) | 11.3 ± 9.9 (1) | 2.5 ± 3.6 (1) | 14.3 ± 13.3 (0) |
| G ($A_2O$) | Anaerobic tank | 173.3 ± 43.7 (0) | 81.2 ± 21.0 (0) | 89.3 ± 26.3 (0) | 48.9 ± 22.2 (0) |
| | Anoxic tank | 124.6 ± 43.3 (0) | 75.5 ± 18.8 (0) | 34.7 ± 19.5 (0) | 14.8 ± 0.8 (0) |
| | Oxic tank | 12.8 ± 17.8 (1) | 145.0 ± 47.1 (0) | 0.1 ± 0.2 (2) | 10.9 ± 5.4 (0) |
| | Final settling tank | 80.2 ± 37.6 (0) | 154.6 ± 77.3 (0) | 0.6 ± 1.0 (2) | 7.3 ± 6.4 (1) |

Data were calculated as 100 × (relative NR agonistic activity after biological treatment tank or final settling tank)/(relative NR agonistic activity after primary settling tank) using the results obtained at concentration factors of 10 (RARα), 50 (ERα) or 100 (TRα and RXRα). Results shown are the means ± SD of three sampling dates.
Numbers in parentheses indicate the frequencies showing 0% agonistic activity during the three sampling events.

at the oxic tank, and reached a level similar to that in the CAS at the final settling tank. Thus, TR agonist(s) in municipal wastewater appeared to be efficiently transformed to less-bioactive forms under aerobic conditions. However, in $A_2O$, the TRα agonistic activity increased markedly in the oxic tank, eventually reaching higher levels than were observed in the influent. This trend was also observed in our initial investigation in WWTP-D, which employed $A_2O$ (Fig. 1c). These findings suggest that the long anaerobic treatment followed by aerobic treatment in the $A_2O$ process may generate some TRα agonistic byproducts. Based on these results, the effectiveness of the evaluated processes on the removal of TRα agonistic activity in wastewater was CAS ⩾ AO > pAO > $A_2O$.

### 2.2.3 Removal of RARα agonistic activity

4-Oxo-atRA and 4-oxo-13-*cis* RA, the major RAR agonists identified in sewage in Beijing, China, are eliminated from human and animal bodies through urinary excretion mainly as glucuronide conjugates (Zhen et al., 2009). However, unlike ERα agonistic activity, RARα agonistic activity did not increase at the first biological treatment tank in most cases, and the increase was observed in only two of a total of 12 samples (one AO sample and one $A_2O$ sample) (Table 2). Therefore, it was suggested that the deconjugation of glucuronide conjugates of 4-oxo-RAs may occur very easily and be completed before inflow into WWTPs in most cases.

RARα agonistic activity declined markedly at the aeration tank in CAS and at the oxic tank in AO and $A_2O$ (Table 2). Conversely, the biological treatment in pAO did not effectively reduce the RARα agonistic activity, as indicated by more than 30% of the activity remaining in the effluent. Among CAS, AO and $A_2O$, the RARα agonistic activity was lower in the effluent of AO and $A_2O$ than in that of CAS. Thus, a combination of aerobic and anaerobic treatments or treatment with long sludge retention time appeared to be favorable for the removal of RARα agonist(s) from wastewater. Overall, the ability of the different treatment systems to reduce the RARα agonistic activity in wastewater was $A_2O$ ≈ AO > CAS > pAO.

### 2.2.4 Removal of RXRα agonistic activity

RXRα agonistic activity of wastewater was reduced by at least 50% in the first biological treatment tank of all four WWTPs (Table 2). Specifically, the activity in the aeration tank in CAS was less than 10% on average and 0% on two of the three sampling dates. These findings suggest that aerobic treatment can reduce the RXRα agonistic activity of wastewater more efficiently than anaerobic treatment. In the WWTPs that employed the other three processes, the removal performance increased with the number of biological treatment tanks (i.e., $A_2O$ > AO > pAO). Although the average remaining RXRα agonistic activity in the effluent of $A_2O$ was similar to that of CAS, the performance seemed to be slightly higher in CAS than $A_2O$ because the activity of the effluent was 0% on two of the sampling dates in CAS. Thus, the overall ability of the four tested treatment processes to reduce the RXRα agonistic activity in wastewater was ranked as CAS ⩾ $A_2O$ > AO > pAO.

## 3 Conclusions

In this study, we examined the agonistic activities of untreated and treated wastewater in municipal WWTPs in Japan against steroidal NRs (ERα) and non-steroidal NRs (TRα, RARα and RXRα) using a yeast two-hybrid assay. The results indicated that municipal wastewater commonly contains EDCs with agonistic activities against both steroidal and non-steroidal NRs. Our results also showed that, although the agonistic activities against NRs declined during the activated sludge treatments in WWTPs, they were not completely removed from the effluent. Thus, EDCs that can interfere with not only steroidal NRs but also non-steroidal NRs are likely to be discharged into the natural aquatic environment. Although preliminary assessment of the potential risks based on the agonistic activity of WWTP effluents against the four tested NRs obtained in the yeast assays suggested that the measured levels of non-steroidal NR agonists in effluent were not likely to cause any biological effect, further study to identify the major compound(s) exhibiting agonistic activity against each of the target non-steroidal NRs is required to assess

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the potential endocrine disruptive effects through those NRs in more detail.

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