SUPPORTING INFORMATION

for

Identification of Retinoic Acid Receptor Agonists in Sewage Treatment Plants

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Chemicals and Standards. All-*trans*-retinoic acid (all-*trans*-RA), 13-*cis*-retinoic acid (13-*cis*-RA), 9-*cis*-retinoic acid (9-*cis*-RA) and [$^2H_9$]Progesterone (PGT-d_9) were purchased from Sigma (St. Louis, MO, USA). All-*trans*-4-oxo-retinoic acid (all-*trans*-4-oxo-RA) and 13-*cis*-4-oxo-retinoic acid (13-*cis*-4-oxo-RA) were obtained from Toronto Research Chemicals (TRC, Toronto, Canada). Methanol, acetonitrile, ethyl acetate, hexane and formic acid were of HPLC grade and purchased from Fisher Chemical (New Jersey, USA) and dimethylsulfoxide (DMSO) was purchased from Sigma (St. Louis, USA). Hydrochloric acid was analytical grade (Beijing Chemicals, China). Ultrapure water was prepared using a compact ultrapure water system (Easypure UV, USA) under a conductivity of 18.2 $\Omega \cdot cm^{-1}$.

Sample Collection. The relative amount of wastewater (effluent) in Tonghui River and Qinghe River were calculated to be 96% and 93%, respectively. The water temperatures during the sampling campaigns were 28 and 9°C in July and January for both rivers, respectively. Unfortunately, we cannot get the number of inhabitants living in the Tonghui River and Qinghe River watershed. Based on the data of water flow in two pipes and Tonghui River, the typical dilution factors from wastewater coming from Pipes 1 and 2 to river waters were calculated to be 235 and 191, respectively.

UPLC ESI-MS/MS. The bioactive HPLC fraction was analyzed by an electrospray ionization tandem mass spectrometry (ESI-MS/MS) using a Quattro Premier XE tandem quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an ACQUITY Ultra Performance LC (Waters, Milford, MA). Separation was conducted exactly under the same condition as UPLC fractionation. Data acquisition was performed in the positive ion mode. The capillary voltage, cone voltage, and multiplier voltage were set at 2.6 kV, 30 V, and 650 V, respectively. The flow of desolvation gas and cone gas were set at 500 and 50 l/h, respectively. The source temperature and desolvation gas temperature were held at 110 and 350 °C, respectively. Argon was used as the collision gas, and the collision energy was set at 15 eV for

acquiring MS/MS spectra.

Quantitation and Quality Assurance/Quality Control (QA/QC). Quantitative analysis for all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA was performed using LC-ESI-MS/MS in multi-selected reaction monitoring (MRM). MS/MS spectra of the parent ion m/z 315 (ESI positive ion mode) of all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA were recorded in the range from 100 to 500 m/z. Figure S2 shows the MS/MS spectra of all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA in the full scan product-ion experiments at the collision energy of 15 eV. For each 4-oxo-RA, the 315 m/z to 137 m/z transition ($[M+H]^+$ to $[M+H-H_2O-CO-C_{10}H_{12}]^+$ at collision energy of 25 eV) was selected for quantitation, and the ratio of the quantitation transition and identification transition (315 m/z to 214 m/z, $[M+H]^+$ to $[M+H-H_2O-CO-CO]^+$ at collision energy 20 eV) was used for confirmation. The injection volume was 5 μL, and the instrumental detection limits for all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA were both 5 pg.

Figure S3 shows the UPLC-MS/MS chromatograms of Gaobeidian STP influent before and after HPLC fractionation. It can be found that the signal/noise (S/N) ratios for all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA were largely improved after HPLC fractionation, indicating that HPLC fractionation was effective in reducing the matrix effects during UPLC-MS/MS analysis. However, stable isotope labeled standards are still desirable to ensure the accuracy of quantification in HPLC-MS/MS analysis of environmental samples considering the matrix effects and the variation of instrument response from injection to injection. Since the stable isotope labeled standards for all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA are not commercially available, PGT-d_9 was selected for a potential internal standard. We spiked PGT-d_9 at concentration of 3.3 μg/l, and all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA at concentration of 71.4 μg/l into extracted influent matrix, and then compared their responses between the spiked matrix and standard solution. The signal suppressions (%) for influent samples were calculated by the following equation (*1*):

Signal suppressions(%) = $1 - (R_{sp} - R_b) / R_s$ (1)

where R_{sp} is the response of spiked compound in the sample extraction, R_b is the response of unspiked sample extraction, and R_s is the response of spiked compound in the standard solution. Similar signal suppressions for all-*trans*-4-oxo-RA (14.8 ± 11.4%, n=3), 13-*cis*-4-oxo-RA (16.3 ± 2.2%, n=3), and PGT-d_9 (13.8 ± 5.3%, n=3) were observed. In addition, PGT-d_9 was eluted at the retention time of 3.96 min, which is similar to the retention times of all-*trans*-4-oxo-RA (3.55 min) and 13-*cis*-4-oxo-RA (4.13 min), and the isocratic conditions used in this study ensured the same ionization condition for PGT-d_9, all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA. Thus, PGT-d_9 was used as an internal standard to determine the concentration of all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA in the samples.

Four points calibration curve was constructed for quantification of all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA. The reference standards used in the calibration were all freshly prepared by spiking 60% acetonitrile solution with all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA at concentrations of 2, 10, 40 and 200 μg/l, and 3.3 μg/l of PGT-d_9 for each concentration.

Recovery experiments were carried out by spiking standard solutions of all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA to ultra pure water, influent and effluent samples from Gaobeidian STP and river water samples. The spiked concentrations were 2 ng/l for ultrapure water, 30 ng/l for influent, 2 ng/l for effluent and 2 ng/l for river water which were at least three times higher than the original concentrations that were determined prior to the fortification experiment. The sample preparation procedure was exactly same as the procedure for detection of all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA in STP and river water samples. The recoveries for all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA were 65.0 ± 1.6% and 59.8 ± 9.6% for ultrapure water, 61.0 ± 0.9% and 53.9 ± 1.4% for influent sample, 57.2 ± 3.6% and 64.8 ± 8.1% for effluent samples and 54.7 ± 4.3% and 61.0 ± 7.1% for river water samples (n=3). Since all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA were expected to occur in STP influents,

effluents and river water, the method detection limits (MDLs) were estimated based on the peak-to-peak noise of the baseline near the analyte peak obtained by analyzing field samples and on a minimal value of signal-to-noise of 3, respectively. The MDLs for all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA were 0.2 ng/l and 0.9 ng/l in influent, 0.2 ng/l and 0.4 ng/l in effluent and 0.2 ng/l and 0.4 ng/l in river water, respectively.

Yeast Assay for RAR Agonistic Activity. The yeast two-hybrid assay described in a previous paper (*2*) was applied to evaluate the RAR-mediated activity of samples. The yeast two-hybrid assay system with three subtypes of retinoic acid receptors, RARα, RARβ and RARγ, and the coactivator, TIF2, was used to investigate the transcriptional activation induced by samples. The yeast cells were preincubated at 30°C for 16 hours in 5 ml medium (6.7 g/l Difco yeast nitrogen base without amino acids, 0.2% glucose, 300 mg/l L-isoleucine, 1500 mg/l L-valine, 200 mg/l L-adenine hemisulfate salt, 200 mg/l L-arginine HCl, 200 mg/l L-histidine HCl monohydrate, 300 mg/l L-lysine HCl, 200mg/l L-methionine, 500mg/l L-phenylalanine, 200 mg/l L-threonine, 300 mg/l L-tyrosine, 200 mg/l L-uracil (Sigma, USA)). 50 µl of overnight culture and 2.5 µl of DMSO solution diluted to the desired concentrations were then added to 200 µl of fresh medium (2% galactose) in a microtube (Axygen Scientific, U.S.A.), respectively. After yeasts were cultured for 4 h at 30°C, 150 µl of the above culture was fractionated, and its absorbance at 595 nm was detected. The residual culture (100 µl) was centrifuged at 4 ºC (15000 rpm) for 5 min, and the collected cells were resuspended in 200 µl of Z buffer (0.1 M sodium phosphate (pH = 7.0), 10 mM KCl, 1 mM $MgSO_4$) containing 1mg/ml Zymolyase 20T (Seikagaku, Tokyo), and incubated for 20 min at 30ºC. The enzymatic reaction was started by the addition of 40 µl of 4 mg/ml 2-nitrophenyl-β-D-galactopyranoside (ONPG, Tokyo Kasei, Tokyo, Japan), and incubated for 20 min at 30ºC. Then the enzymatic reaction was stopped by adding 1 M Na_2CO_3 (100 µl). After the above solution was centrifuged, 150-µl aliquots were placed into 96 wells of a

microplate. Absorbances at 415 and 570 nm were read on a microplate reader (Bio RAD 550, USA) to estimate the RAR-mediated activity, and the β-galactosidase activity (U) was calculated according to Equation (2):

$$U=1000\times([OD_{415}]-[1.75\times OD_{570}]/([t]\times[v]\times[OD_{595}]) \quad (2)$$

where t represents the reaction time (min); v is the volume of the culture used in the assay (ml); OD_{595} is the cell density at the start of the assay; OD_{415} is the absorbance by o-nitrophenol at the end of the reaction, and OD_{570} is the light scattering at the end of the reaction. In this assay, all-*trans*-RA was used as positive control, and the sample response of β-galactosidase activity were expressed as a percentage of the maximum response observed for standard curves developed on the same day (% all-*trans*-RA Max). The ±3 standard derivation (SD) from the mean solvent control response (set to 0% All-*trans*-RA Max) was defined as the significant line in the RAR mediated bioassay. The molar concentration for each retinoid in well of a microplate that produces 50% (EC_{50}) of the maximum response of corresponding RAR agonistic activity was calculated by the Prism 4 for Windows program (GraphPad Software, Inc.).

Yeast Assay for Inhibition of RAR agonistic Activity. A similar assay was used to test the inhibition of RAR agonistic activity by measuring the ability of the environmental samples to inhibit β-galactosidase induction by all-*trans*-RA. The RAR agonistic activity of 2.5 μl of DMSO standard solution containing 50 μg/l all-*trans*-RA and 2.5 μl of DMSO solution of a sample (influents, effluents and river water) which was added by 50 μg/l all-*trans*-RA were detected following the same method as described in the assay for RAR agonistic activity. The β-galactosidase activity was converted to percentage inhibition according to Equation 3.

$$\text{Inhibition } (\%) = (Unit_{max} - Unit_x)/Unit_{max}\times 100 \quad (3)$$

where $Unit_{max}$ = β-galactosidase activity of 50 μg/l all-*trans*-RA in DMSO and $Unit_x$ = β-galactosidase activity of 50 μg/l all-*trans*-RA in mixed samples of F1, F2 and F3 from

influents, effluents or river water.

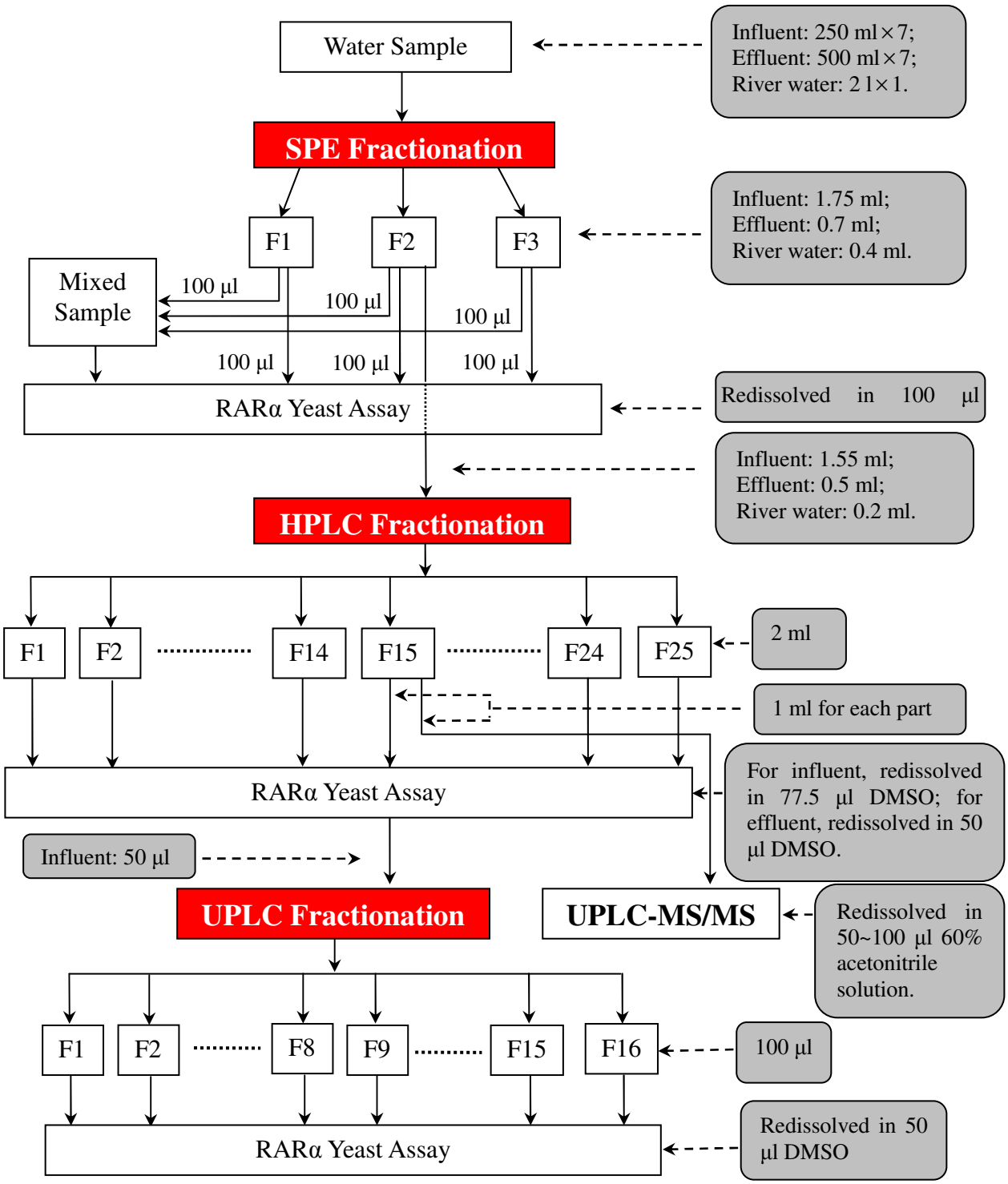


FIGURE S1. Sample preparation procedure for the identification of RARα agonist in Sewage Treatment Plant.

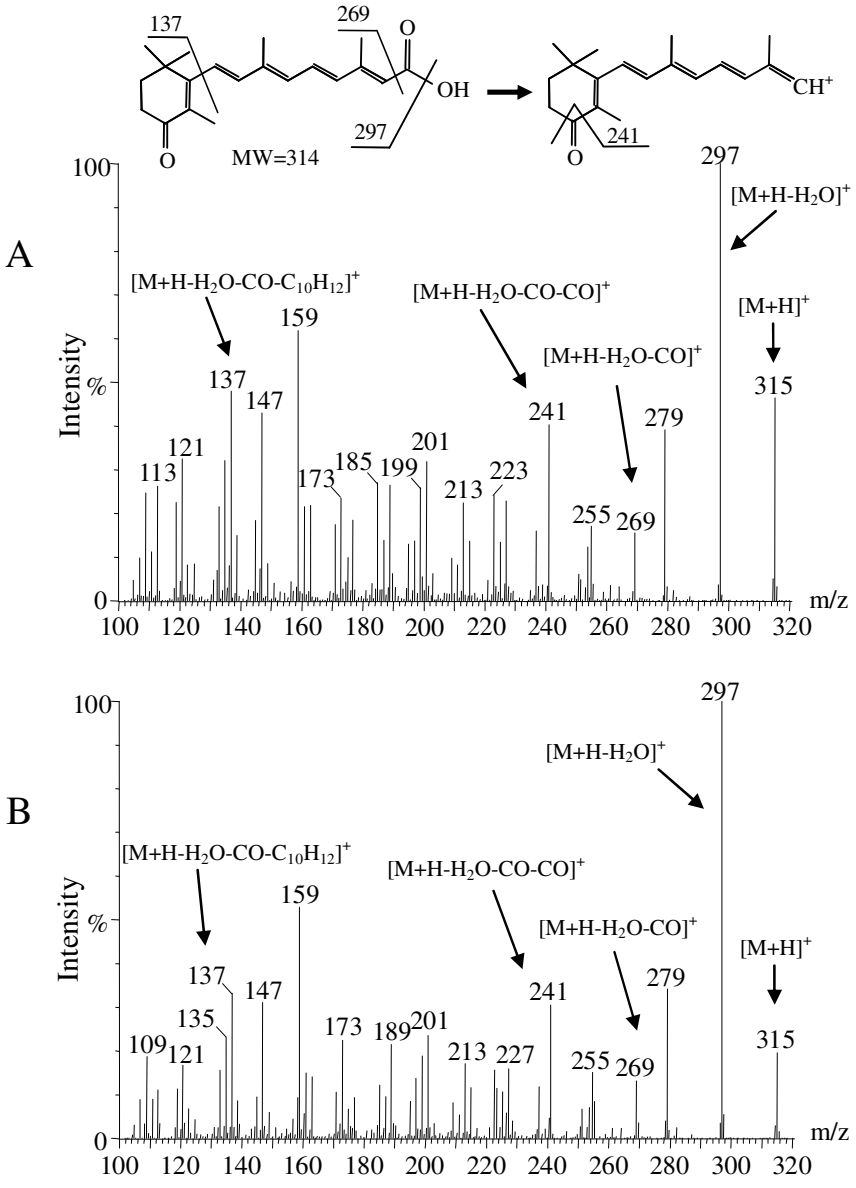


FIGURE S2. MS/MS spectra of (A) all-*trans*-4-oxo-RA and (B) 13-*cis*-4-oxo-RA at a collision energy of 15 eV.

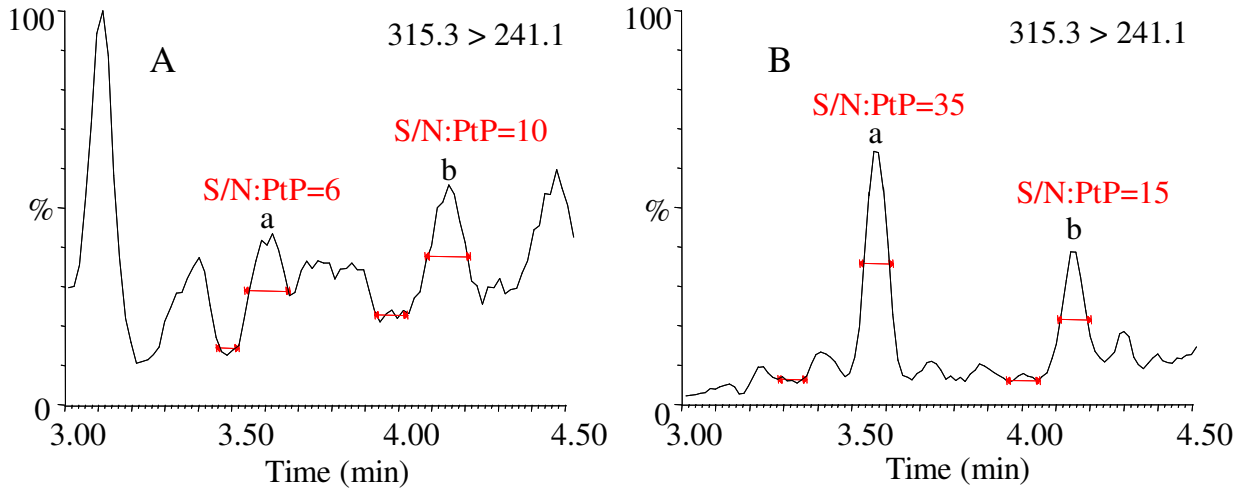
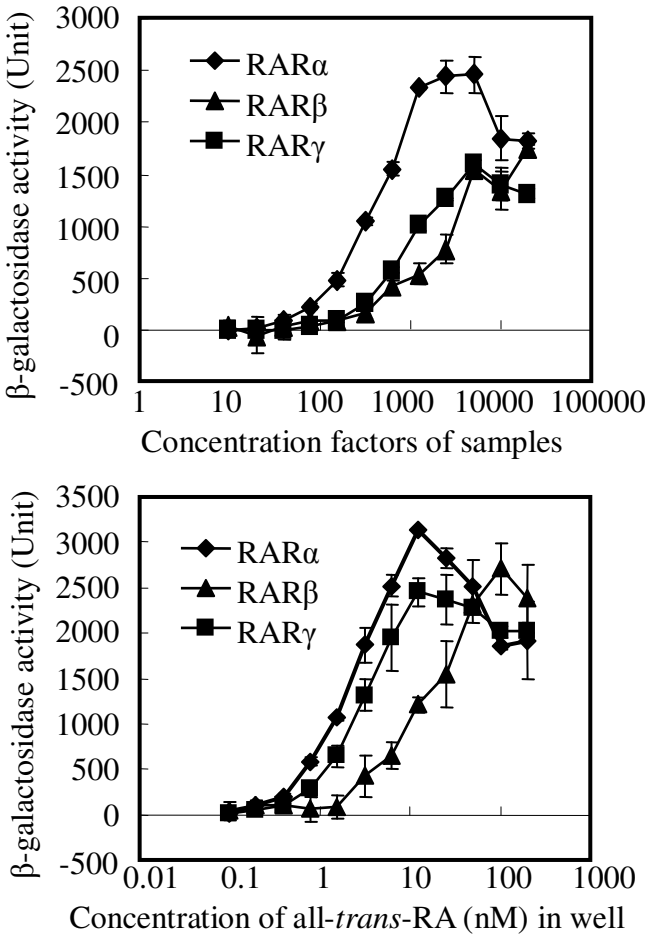


FIGURE S3. Comparison of the UPLC-MS/MS chromatograms of Gaobeidian influent before (A) and after (B) HPLC fractionation. Peak a and b represent all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA, respectively.



158
159 **FIGURE S4**. RAR agonistic activities (α, β, γ) of the HPLC F15 (up panel) from a
160 wastewater sample in comparison with all-*trans*-RA (down panel). Concentration factors of
161 samples in up panel are calculated by dividing the original water volume by the volume of
162 concentrated samples which are used for RAR agonistic activity test.

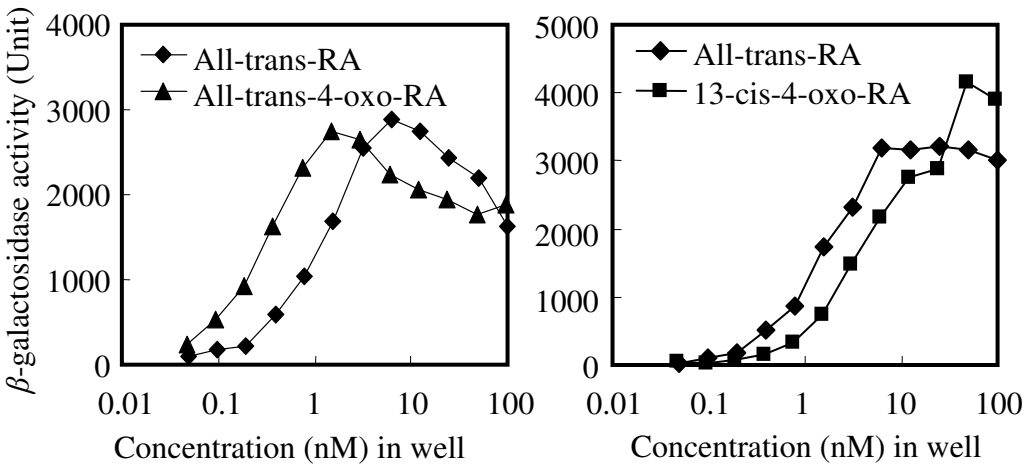


FIGURE S5. Dose-response curves of all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA in comparison to all-*trans*-RA in RARα yeast two-hybrid assay.

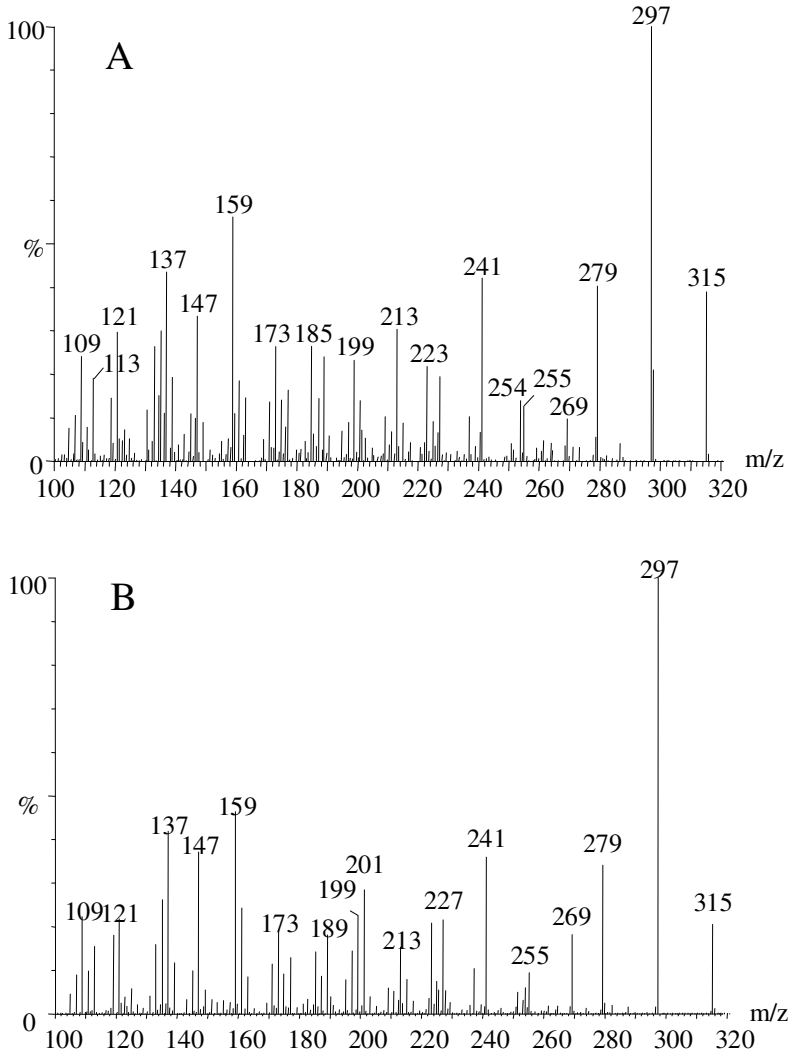


FIGURE S6. MS/MS spectra of the base peak ion of m/z 315 (ESI positive ion mode) in STP influent sample at collision energy of 15 eV. (A) Retention time at 3.55 min; (B) Retention time at 4.13 min.

TABLE S1. Some Available Technical Characteristics of the Six Activated Sludge Sewage Treatment Plants (STP) Considered in This Study*

STP	Inhabitants	Loading (m^3/day)	HRT[a] (h)	SRT[b] (d)	TSS[c,f] (mg/l) in/out	BOD_5[d,f] (mg/l) in/out	COD_{cr}[e,f] (mg/l) in/out
Beixiaohe	400,000	60,000	6-7	5.2	199/12	165/9	341/40
Fangzhuang	100,000	40,000	9.7	10	344/11	295/9	612/38
Gaobeidian	2400,000	791,500	9	10-12	278/10	159/8	329/34
Jiuxianqiao	480,000	200,000	8-11	10.2-18	170/11	154/9	318/38
Qinghe	814,000	474,300	13.5	12.2-17	299/11	194/9	404/40
Wujiacun	180,000	15,000	7	11	116/11	104/9	211/39
Xiaohongmen	1925,000	600,000	12.5	12	355/13	221/10	454/44

*all STPs contained anaerobic, anoxic and aerobic process excepte Beixiaohe STP, which only contained the latter two processes.

a) HRT= hydraulic residence time; b) SRT= solid residence time; c) TSS= total suspended solids; d) BOD_5= five-day biochemical oxygen demand; e) COD_{cr}= chemical oxygen demand consumption using the dichromate method. f) averaged values during this study.

177 **TABLE S2.** ATRA-EQ (ng/l) of three fractions in STP influents and effluents [a]

	A [b]	B	C	D	E	F	G
				Influent			
F1	nd [c]	6.5	nd	5.8	nd	nd	nd
F2	13.1	10.4	10.9	11.2	13.4	6.6	10.5
F3	nd	nd	nd	nd	nd	nd	nd
Mixed	nd	nd	nd	nd	nd	nd	nd
				Effluent			
F1	nd	nd	nd	nd	nd	nd	nd
F2	1.7	2.9	3.2	1.0	1.2	0.9	0.9
F3	nd	nd	nd	nd	nd	nd	nd
Mixed	0.9	1.6	1.9	0.8	1.0	0.7	nd

178 a) LOQ is 0.5 ng/l (ATRA-EQ) in RARα Yeast Assay.
179 b) A: Gaobeidian; B: Beixiaohe; C: Fangzhuang; D: Xiaohongmen; E: Wujiacun; F: Jiuxianqiao;
180 G: Qinghe.
181 c) nd= no detection.

TABLE S3. ATRA-EQ (ng/l) of three fractions in Tonghui River in summer and winter [a]

	upstream 2 km	gaobeidian STP effluent	downstream 0.5 km	downstream 0.55 km	downstream 2.55 km	downstream 2.6 km
			Summer (2006/7/2)			
F1	nd [b]	nd	nd	nd	nd	ND [c]
F2	3.0	1.7	2.9	5.7	3.5	ND
F3	nd	nd	nd	3.3	nd	ND
Mixed	2.6	1.6	2.2	4.9	2.3	ND
			Winter (2007/1/2)			
F1	nd	nd	nd	nd	nd	nd
F2	1.7	1.1	1.9	7.1	2.5	8.3
F3	nd	nd	nd	nd	nd	nd
Mixed	nd	nd	nd	nd	nd	nd

a) LOQ is 0.5 ng/l (ATRA-EQ) in RARαYeast Assay.

b) nd = no detection.

c) ND= no data.

TABLE S4. ATRA-EQ (ng/l) of three fractions in Qing River in summer and winter [a]

	upstream 4 km	upstream 2 km	Qinghe STP effluent	downstream 2 km	downstream 4 km
			Summer (2006/7/2)		
F1	nd [b]	nd	nd	nd	nd
F2	10.0	4.2	0.9	4.0	2.6
F3	11.2	4.0	nd	nd	nd
Mixed	11.6	4.7	nd	2.1	nd
			Winter (2007/1/2)		
F1	nd	nd	nd	nd	nd
F2	5.1	2.9	0.8	0.7	1.2
F3	nd	nd	nd	nd	nd
Mixed	2.2	1.2	nd	nd	0.5

a) LOQ is 0.5 ng/l (ATRA-EQ) in RARα Yeast Assay.

b) nd= no detection.

TABLE S5. Concentrations (ng/l) of all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA, and ATRA-EQ_{cal} of samples in Tonghui River

	upstream 2 km	Gaobeidian STP effluent	downstream 0.5 km	downstream 0.55 km	downstream 2.55 km	downstream 2.6 km
			Summer (2006/7/2)			
all-*trans*-4-oxo-RA	0.7	0.5	0.3	0.9	0.8	ND[a]
13-*cis*-4-oxo-RA	1.4	0.4	1.4	1.6	1.4	ND
ATRA-EQ_{cal}	3.1	2.0	1.7	4.0	3.6	ND
			Winter (2007/1/2)			
all-*trans*-4-oxo-RA	nd[b]	0.5	0.7	0.9	0.4	1.6
13-*cis*-4-oxo-RA	0.8	nd	0.4	0.7	0.3	1.5
ATRA-EQ_{cal}	0.7	1.9	2.7	3.6	1.6	6.7

a) ND= no data.
b) nd = no detection.

193 **TABLE S6.** Concentrations (ng/l) of all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA, and ATRA-EQ_{cal}
194 of samples in Qing River

	upstream 4 km	upstream 2 km	Qinghe STP effluent	downstream 2 km	downstream 4 km
			Summer (2006/7/2)		
all-*trans*-4-oxo-RA	1.0	0.7	0.2	0.3	nd[a]
13-*cis*-4-oxo-RA	0.7	0.7	0.4	nd	nd
ATRA-EQ_{cal}	4.0	2.9	0.9	1.3	0.5
			Winter (2007/1/2)		
all-*trans*-4-oxo-RA	1.8	0.8	nd	nd	nd
13-*cis*-4-oxo-RA	1.1	0.8	nd	nd	nd
ATRA-EQ_{cal}	7.1	3.2	0.5	0.5	0.5

195 a) nd = no detection.

Literature Cited

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(2) Nishikawa, J.; Saito, K.; Goto, J.; Dakeyama, F.; Matsuo, M.; Nishihara, T. New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. *Toxicol. Appl. Pharmacol.* **1999**, *154*, 76-83.