Original Article

Analysis of Environmental Endocrine Disrupting Activities in Wastewater Treatment Plant Effluents Using Recombinant Yeast Assays Incorporated with Exogenous Metabolic Activation System*

LI Jian[1,#], CHEN Ming[1], WANG ZiJian[2], MA Mei[2], and PENG XianZhi[3]

1. Engineering Research Center of Groundwater Pollution Control and Remediation, Ministry of Education, College of Water Sciences, Beijing Normal University, Beijing 100875, China; 2. State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China; 3. State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, Guangdong, China

Abstract

Objective To measure the endocrine disrupting chemicals (EDCs) in wastewater and evaluate the EDCs removal efficiencies in the municipal wastewater treatment plants (WWTP).

Methods A battery of *in vitro* recombinant yeast bioassays incorporated with exogenous metabolic activation system (rat liver preparation, S9 mix) was conducted to assess the estrogen receptor (ER), androgen receptor (AR), progesterone receptor (PR), and thyroid receptor (TR) ant/agonistic activities of effluents collected from Datansha WWTP.

Results The indirect estrogenic, anti-androgenic, anti-progesteronic, and anti-thyroidic activities were observed in the influent. The removal efficiencies of EDCs were above 74%, suggesting that the present wastewater treatment processes were good enough to remove most of these indirect endocrine disrupting chemicals.

Conclusion The incorporation of exogenous metabolic capacity into the test system was valid for the study of indirect effects on ER, AR, PR, and TR.

Key words: Recombinant yeast assay; Endocrine disrupting chemicals; Wastewater sample; Metabolic activation

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INTRODUCTION

Over the last 20 years there has been increasing concern within the public and scientific world regarding numerous environmental chemicals, called endocrine disrupting chemicals (EDCs), which can modulate the neuro-endocrine system and thus adversely affect human and wildlife reproduction[1]. Nuclear receptor (NR) superfamily consists of a large group of receptors that are involved in the regulation of a wide range of physiological functions in eukaryotic organisms[2]. Numerous interactions of EDCs with the signaling pathway of NRs were previously studied. Several possible mechanisms of action, including direct hormone agonists or antagonists that could mimic or inhibit endogenous hormone activities have been suggested[3]. Thus, NRs have become one of the research subjects in endocrine disruptors.

It has been suggested that industrial/municipal effluents and urban/agricultural runoff are important sources of EDCs discharged into the aquatic environment[4]. In particular, many recent studies have demonstrated that wastewater treatment plant (WWTP) effluents can affect the

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#Correspondence should be addressed to LI Jian. Tel: 86-10-58802738. Fax: 86-10-58802738. E-mail: lijian@bnu.edu.cn

Biographical note of the first author: LI Jian, female, born in 1980, lecture, majoring in aquatic ecotoxicology.

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development of fish embryos and larvae[5] and are important point resources for residues of EDCs in the aquatic environment[6]. It is necessary to investigate the endocrine disrupting activities in WWTP effluents and to evaluate the detoxication efficiencies of wastewater treatment processes. However, previous studies has focused primarily on estrogen receptor (ER) agonists of the WWTP effluents but ignored other potential EDCs target sites[7]. To accurately predict the potential disrupting effects of the WWTP effluents, the interaction of EDCs with other NRs, such as androgen receptors (ARs), progesterone receptors (PRs), and thyroid receptors (TRs), should also be studied.

Recent studies have demonstrated that several chemicals may exert anti-androgenic effect by interfering with ARs, which play an important role in male mammals[8]. For example, *p,p*′-dichlorodiphenylethane (*p,p*′-DDE), bisphenol A (BPA), octylphenol and nonylphenol have been demonstrated *in vitro* AR-mediated anti-androgenic activities[9]. Large amount of these chemicals were also detected in wastewater[10]. Secondly, progesterone is a key regulator of proliferation and differentiation in reproductive tissues[11]. Several pieces of evidence indicate that some synthetic chemicals in the environment can inhibit the binding of the progesterone to PR[12]. Thirdly, thyroid hormone is essential for normal brain development in both humans and animals. More and more evidence from animal and *in vitro* studies demonstrate that the thyroid is vulnerable to endocrine-disrupting effects[13]. Industrial chemicals reported to be the major thyroid hormone disruptors include polychlorinated biphenyls (PCBs), dioxins, flame-retardants, phenols, and phthalates[14]. Currently, there is a need for valid and rapid methods to detect the endocrine disrupting effects of WWTP effluents at the level of these NRs.

The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) outlined a tiered screening/testing strategy for EDCs. Included in the Tier I testing scheme are *in vitro* transactivation assays (e.g. yeast-based bioassays) to screen for chemical substances and mixtures that interact with the estrogen, androgen, and thyroid hormone/receptor systems[15]. Currently, the yeast-based bioassays for the determination of estrogenic, androgenic and progesteronic activities are mainly used for environmental monitoring for research purposes[7,14,16]. Yeast cells have several advantages, including fast growth, easy handling, cheap media components, and robustness toward toxic effects of test chemicals or solvents. Thus, yeast-based bioassays are a fast and easy tool for monitoring the endocrine disrupting activity of environmental samples prior to subjecting them to more expensive and time-consuming *in vivo* tests. However, one of the limitations of these *in vitro* assays is their limited metabolic capacity[17]. In order to overcome this limitation, Morohoshi[18] reported the incorporation of an exogenous metabolic activation system (rat liver preparation, S9 mix) into the yeast two-hybrid estrogen assay. EDSTAC also recommended that the evaluation of chemicals *in vitro* should be performed in the presence and absence of metabolic activity to enhance the chances of detecting compounds with prohormonal activities[15].

In this study, the recombinant yeast assays incorporated with exogenous metabolic activation system (rat liver preparation, S9 mix) were carried out to investigate the effects of WWTP effluents on ER, AR, PR, and TR-mediated transcription of β-galactosidase *in vitro* in reporter yeasts. All samples were treated with S9 mix to better detect the possible effects of mammalian metabolic activation/deactivation of the EDCs. These test systems could be used for the rapid, high-throughput screening of samples for their indirect agonist and antagonist properties for ER, AR, PR, and TR, and for assessing the removal efficiencies of treatment processes in the WWTP.

MATERIAL AND METHODS

Sample Collection and Processing

Wastewater samples were collected from Datansha wastewater treatment plant in Guangzhou, China in December 2006. The Datansha WWTP with a capacity of 165 000 m^3/d mainly treats residential sewage (90%) and serves for 1.5 million populations. This plant was constructed for reclamation of the wastewater utilizing two trains. These trains adopt the identical treatment steps, including grid, sand filter, anaerobic tank, anoxic tank, aerobic tank, clarifier, and chlorination. Samples were taken from influent (A), effluent after sand filter treatment (B), effluent from train1 (C), and effluent from train2 (D). Grab samples (10 L) were collected. Appropriate amount of solvent-cleaned sodium azide (500 mg/L) was added in each sample right after sampling to suppress possible biotic activities. Samples were stored at 4 °C and treated within 48 h[19]. Water

samples (5 L) were extracted by solid phase extraction (SPE) using Oasis C18 cartridges (6 mL, 1g, Waters), eluted with the mixture of methanol and acetyl acetate (1:1). Then the raw extracts were filtered by anhydrous sodium sulphate to remove water. This was followed by blowing it to dryness under a nitrogen stream. The residues were dissolved in 0.5 mL dimethyl sulfoxide (DMSO, Sigma, USA) and diluted by a 0.5-fold dilution series.

Yeast Strain

Yeast strains specifically transformed with the ER, AR, and TR gene were constructed in our laboratory and grown in synthetic dextrose (SD) medium (lacking tryptophan and leucine, SD/-Trp/-Leu)[14,20]. Another yeast strain transformed with the PR gene was a kind gift of Dr. Kevin W. Gaido (Chemical Industry Institute of Toxicology, Research Triangle Park, North Carolina, USA), which was grown in synthetic complete (SC) medium with tryptophane, but lacking histidine and leucine (SC/-His/-Leu)[21].

β-Galactosidase Assay

In performing the assay, cultures in the exponential growth phase (grown overnight) were diluted with SD/-Leu/-Trp or SC/-His/-Leu medium to an OD_{600} of 0.75[20]. All assays were conducted in at least triplicate. Each triplicate included a positive control and a negative control (DMSO). The S9 mix which was prepared from livers of male Sprague-Dawley rats pretreated with 3-methylcholanthrene and phenobarbital[22], water and cofactor ($MgCl_2 \cdot 6H_2O$, KCl, G-6-P, nicotinamide adenine dinucleotide phosphate, nicotinamide adenine dinucleotide, Na_2HPO_4 and NaH_2PO_4) was added to the SD/-Leu/-Trp or SC/-His/-Leu medium[23]. 5 μL of serial dilutions of test samples were combined with 5 μL of S9 mix and 990 μL of medium containing 5×10^3 yeast cells/mL resulting in a test culture in which the volume of DMSO did not exceed 1.0% of the total volume. Two hundred microliters of the test cultures were transferred into each well of the 96-well plate (Costar, NY, USA) and incubated at 30 °C with vigorous orbital shaking (800 rpm) on a titer plate shaker (Heidolph TITRAMAX 1000, Hamburg, Germany) for 2 h, then the cell density of the culture was measured at 600-nm wavelength (TECAN GENios A-5002, Salzburg, Austria). A 50 μL test culture was transferred to a new 96-well plate and after addition of 120 μL of Z-buffer (16.1 g/L $Na_2HPO_4 \cdot 7H_2O$; 5.5 g/L $NaH_2PO_4 \cdot H_2O$; 0.75 g/L KCl; 0.246 g/L $MgSO_4 \cdot 7H_2O$) and 20 μL chloroform, the assays were carefully mixed (vortex 25 s) and preincubated for 5 min at 30 °C. The enzyme reaction was initiated by addition of 40 μL o-nitrophenyl-β-D- galactopyranoside (13.3 mmol/L, dissolved in Z-buffer). The assays were incubated at 30 °C on a titer plate shaker. The reactions were terminated by the addition of 100 μL Na_2CO_3 (1 mol/L). For the samples, the incubated time lasted for 60 min. After centrifugation at 12 000 g for 15 min (Sigma Laborzentrifugen 2K15, Osterode, Germany), 200 μL of the supernatant was transferred into a new 96-well plate and the OD_{420} was determined. The β-galactosidase activity was calculated according to the report by Gaido[21] and Li[20]. All samples were evaluated in triplicate and β-galactosidase activities of the liquid yeast cultures are expressed as the means and standard deviations. The antagonistic activity of samples was tested by co-incubation of yeast strain with nature ligand as shown in Table 1. The method using ligand in combination with tested chemicals has been widely used to screen the antagonistic activity of ER, AR, and PR[12]. To ensure that increased/reduced activities in the bioassay were caused by true agonistic/antagonistic responses and not by cytotoxicity, viability was measured in cells exposed to samples at the maximum assay concentration. Yeast cells were plated as in the original assay, and then exposed for 2 h to exposure medium (cell-specific medium containing samples). And cell viability was determined spectrophotometrically as a change of cell density (OD_{600}) in the assay medium. In all the bioassays, the procedural blank, which had been subjected to SPE, was also run alongside the samples to monitor for a false-positive result.

Data Analyses

In order to evaluate the effects of samples, the toxicity equivalent method was used. For example, the estradiol equivalents (EEQ) values were calculated to evaluate the effects on ER, comparing the estrogenic activity of the sample extracts with the series concentrations of E_2 standard[24]. Other equivalents were also calculated to evaluate the effects of samples on AR, PR, and TR (Table 2).

RESULTS AND DISCUSSION

Estrogenic Activity

To test the indirect estrogenic activities in WWTP effluents, yeast strain was incubated with the water extractions and S9 mixture as suggested above.

Estrogen receptor agonistic activities were detected in all water samples (Figure 1). The concentration of E_2 equivalent (EEQ) ranged from 1.70 $\times 10^{-9}$ to 1.17$\times 10^{-8}$ g E_2 equivalents /L (Table 2). These values showed high estrogenic activity in the WWTP influent water sample (Figure 1), which was similar to that reported by Céspedes[25] and Svenson[26]. In the effluents, the EEQs were no more than 3.0$\times 10^{-9}$ g E_2 equivalents/L, suggesting most (more than 74%) estrogenic activity might be removed by WWTP treatment. Comparing the residual levels of estrogenic activity in effluents. to the values reported for other countries, we concluded

Table 1. Overview of the Four *in vitro* Bioassays Used to Determine the Possible Endocrine Disrupting Potency on 7 Different Endpoints.

Endocrine Pathway	Bioassay	Reference Material	Endpoint	REC50 or RIC50
Estrogenic	Two-hybrid ER-Laz	E_2	Estrogenic activity through ER	2.5$\times 10^{-10}$ mol/L[20]
Androgenic	Two-hybrid AR-Laz	DHT	Androgenic activity through AR	1.3$\times 10^{-8}$mol/L[20]
		Flutamide	Anti-androgenic activity through AR in the presence of DHT	9.8$\times 10^{-6}$ mol/L[20]
Progestagenic	PR-Laz	Progesterone	Progestagenic activity through PR	5.0$\times 10^{-10}$ mol/L[20]
		RU486	Anti-progestagenic activity through PR in the presence of progesterone	6.6$\times 10^{-6}$mol/L[20]
Thyroidal	Two-hybrid TR-Laz	T_3	Thyroid hormone-mimicking (T_3 like) activity through TR	1.1$\times 10^{-7}$ mol/L[14]
		Amiodarone Hydrochloride	Anti-thyroidic activity through TR in the presence of T_3	2.4$\times 10^{-7}$ mol/L[14]

Note. ER=estrogen receptor, AR=androgen receptor, PR=progesterone receptor, TR=thyroid receptor, E_2=17β-Estradiol, DHT=dihydrotestosteron, T_3=3,3',5-triiodo-L-thyronine, REC50=the concentration inducing 50% of the maximum effect, RIC50=the concentration causing a 50% inhibition of the maximum effect.

Table 2. Endocrine Disrupting Potency of Effluents Collected from Datansha Waste Water Treatment Plant Tested by a Battery of *in vitro* Bioassays Incorporated with Exogenous Metabolic Activation System (Rat Liver Preparation, S9 mix)

Water Sample	*In vitro* Potency						
	ER Agonists	AR Agonists	AR Antagonists	PR Agonists	PR Antagonists	TR Agonists	TR Antagonists
	g E_2/L	g DHT/L	g flutamide/L	g progesterone/L	g RU486/L	g T_3/L	g amiodarone hydrochloride /L
A	1.17E-08	ND	7.34E-05	ND	2.62E-04	ND	1.75E-03
B	3.20E-09	ND	3.36E-05	ND	8.16E-05	ND	5.21E-04
C	3.00E-09	ND	ND	ND	1.52E-05	ND	ND
D	1.70E-09	ND	ND	ND	2.92E-05	ND	ND

Note. A=extract of influent, B=extract of effluent after sand filter treatment, C=extract of effluent from train1, D=extract of effluent from train2, ER=estrogen receptor, AR=androgen receptor, PR=progesterone receptor, TR=thyroid receptor, E_2=17β-Estradiol, DHT=dihydrotestosteron, T_3=3,3',5-triiodo-L-thyronine, ND=not detection.

that these values were comparable with those reported for effluents from Spain sewage treatment plants[25] and those reported in effluents from Norwegian North Sea oil production platforms[16]. However, the levels in this study were lower than those reported for two WWTPs in southern Germany (34.1-65.96 ng/L)[27].

In contrast to data from the present study, in a previous study, the estrogenic activities of these extracted water samples without metabolism had been detected[28]. A comparison of with and without metabolism, the estrogenic activities with metabolism were clearly higher than those without metabolism (1.0-10.1 ng/L). The EEQ value of sample D after metabolism was proved to be about 1.7 times higher than that without metabolism, indicating many proestrogenic chemicals in the waste water have estrogenic activity in the presence of metabolic activity. For example, BPA and methoxychlor are typical proestrogenic chemicals that are metabolically activated in terms of estrogenicity under the conditions existing rat liver S9. Yoshihara[29] reported that the estrogenic activity of BPA was increased by about two to five times after being incubated with rat liver S9. Charles[30] also found the increased methoxychlor response in

the presence of S9. Therefore, the metabolic activation by S9 must be taken into account in order to assess proestrogenic chemicals as an *in vivo* estrogen and evaluate the estrogenic activity of environmental samples. However, the incorporation of metabolic activation into *in vitro* bioassays used for evaluating the endocrine disrupting activities of environmental samples was previously limited. We attempted to incorporation of S9 into recombinant yeast assays to screen ER, AR, PR, and TR disrupting activities. This might be a useful tool for detection of potential endocrine disrupting activities by allowing for an enhanced spectrum of metabolism.

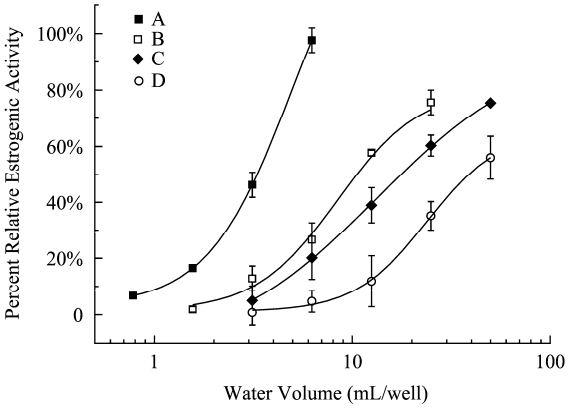

Figure 1. Estrogen receptor (ER) agonistic activities of water extracts determined by the ER yeast bioassay incorporated with exogenous metabolic activation system (rat liver preparation, S9 mix). The samples were collected from Datansha waste water treatment plant and the sample's agonistic activity is represented as the percent induction activity relative to the maximum induced by 17β-Estradiol (E_2, 5×10^{-10} mol/L).Values are presented as the average± standard error (*n*=3). A=extract of influent, B=extract of effluent after sand filter treatment, C=extract of effluent from train1, D=extract of effluent from train2.

(Anti)Androgenic Activity

To study AR agonistic activity after metabolism, AR yeast was tested in samples and S9 mixture with increasing concentrations. None of the samples showed AR agonistic activity (Table 2). However, samples A and B, were found AR antagonistic activities and the dose–response curves were also obtained (Figure 2). Flutamide equivalent concentrations could be determined to be from 3.36×10^{-5} to 7.34×10^{-5} g flutamide equivalents (Table 2). The variations of flutamide equivalent concentrations in treatment processes could be observed clearly, suggesting that these processes removed more than 99% of effect. All of the results indicated the high efficiency of the present treatment processes to remove anti-androgenic chemicals. Compared with sample A, the flutamide equivalent of sample B decreased greatly, suggesting that the sand filter treatment could remove 54% of anti-androgens. The flutamide equivalents of sample C and D were below the detection limit, suggesting that the combination of biological treatment (an/aerobic tank), clarifier and chlorination were good enough to remove anti-androgens.

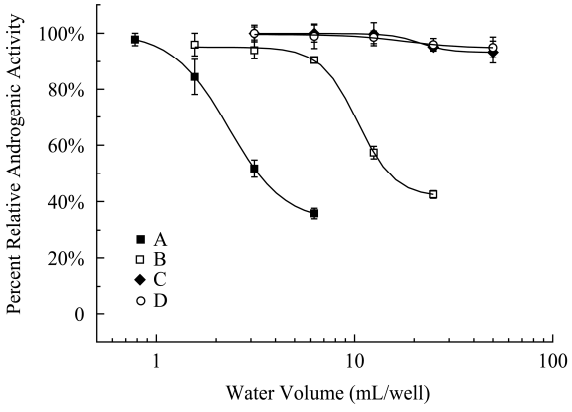

Figure 2. Androgen receptor (AR) antagonistic activities of water extracts determined by the AR yeast bioassay incorporated with exogenous metabolic activation system (rat liver preparation, S9 mix). The samples were collected from Datansha waste water treatment plant and the sample's antagonistic activity is represented as the percent induction activity relative to the maximum induced by dihydrotestosterone (DHT, 5×10^{-8} mol/L). Values are presented as the average ± standard error (*n*=3). A=extract of influent, B=extract of effluent after sand filter treatment, C=extract of effluent from train1, D=extract of effluent from train2.

In this study, we found that the occurrence of AR antagonists, not agonists, in waste water effluents. Tollefsen[16] reported a similar result and found AR antagonists in effluents from Norwegian North Sea oil production platforms. But, the concentrations in our study were lower than those (flutamide equivalent concentrations were 208-344 μg /L) determined in WWTP effluents in the United Kingdom[31]. Limited data have been published on

the anti-androgenic activity of environmental contaminants. The (anti)androgenic effects of bisphenol A diglycidyl ether, bisphenol F diglycidyl ether and their derivatives were studied. Previous results demonstrated that none of these chemicals exhibited androgen agonistic activity, and bisphenol A diglycidyl ether, bisphenol F diglycidyl ether, BPA and bisphenol F had obvious antagonistic activities for androgen[32]. Furthermore, some environmental estrogens were also found to be anti-androgens[8]. For example, environmental xenoestrogen such as vinclozolin, DDE, dichlorodiphenyltrichloroethane (DDT), 4-*t*-octylphenol and 4-phenylphenol had all been shown to be anti-androgens[8].

The AR antagonistic activities after metabolism were higher than those without metabolism (nd-3.4×10^{-5} g/L) reported previously[28]. Especially, the flutamide equivalent of sample A with metabolism was about 2 times higher than that without metabolism[28], indicating S9 could be useful to screen the proandrogenic chemicals existed in the waste water. Rijk[33] reported the bovine liver S9 fraction combined with the androgen-specific yeast bioassay to mimic the *in vivo* metabolic activation and detect androgenic activity of some chemicals. Peters[34] also used the S9 pre-treatment for proandrogens to identify the androgenic chemicals in herbal mixtures and sport supplements. However, these reports focused mainly on proandrogenic chemicals, the proandrogenic activities of environmental samples have rarely been reported so far. WWTP was considered as a major source of EDC-rich pollution. When the effluent with proandrogenic activity discharged into the aquatic environment, the majority of the effects observed in the aquatic environment affect the reproductive system, for instance, the feminization of male fish, and the reproductive abnormalities in fish[33].

(Anti)Progesteronic Activity

The potential activities of samples via PR after metabolic activation were investigated using recombined PR yeast. The results showed that none of the above-mentioned waste water extracts exhibited PR agonistic activity (Table 2). Antagonist activity was determined by co-incubation with 1×10^{-9} mol/L progesterone. All samples showed strong antagonistic effects with RU486 equivalent concentrations above 1.52×10^{-5} g/L (Table 2) and obviously decreased the β-galactosidase expression (Figure 3). It was also found that there was an effective elimination of PR antagonists after treatment. The removal efficiencies were above 88%.

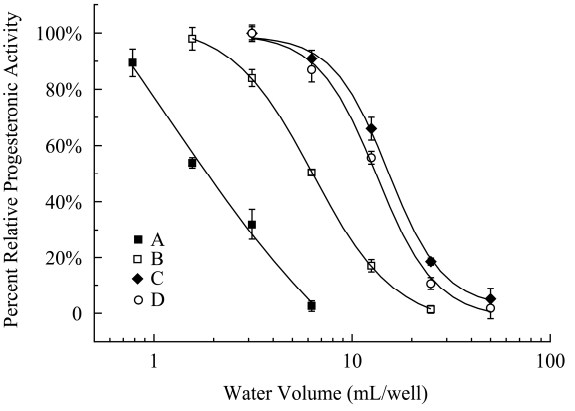

Figure 3. Progesterone receptor (PR) antagonistic activities of water extracts determined by the PR yeast bioassay incorporated with exogenous metabolic activation system (rat liver preparation, S9 mix). The samples were collected from Datansha waste water treatment plant and the sample's antagonistic activity is represented as the percent induction activity relative to the maximum induced by progesterone (1×10^{-9} mol/L). Values are presented as the average ± standard error (*n*=3). A=extract of influent, B=extract of effluent after sand filter treatment, C=extract of effluent from train1, D=extract of effluent from train2.

Progesterones act as reproductive pheromones in fish at extremely low concentrations. Although research on (anti)progesteronic compounds is just at early stage, the presence of (anti)progesteronic contaminants in the aquatic environment has been reported. In this study, the fact that waste water extracts after metabolism inhibited of PR-controlled β-galactosidase expression suggested that some indirect PR antagonists existed in the influent of WWTP. Anti-progesteronic properties of leather industry effluents were reported by Chatterjee[35]. Some organochlorine pesticides and phenolic compounds, such as 4-nonylphenol, hexachlorobenzene, DDT, 4-*t*-octylphenol and pentachlorophenol, were previously found to be PR antagonists[20,36]. Chatterjee[35] further demonstrated that some pesticides and their metabolites had anti-progesteronic activities. Therefore, further work in the analysis of extracts is needed to identify the responsible anti-progesteronic components.

(Anti)Thyroidal Activity

Over recent years, several classes of environmental contaminants or their metabolites have been found to have the abilities to alter thyroid hormone homeostasis through interference with the thyroid hormone signal transduction pathway[13]. In this study, we evaluated the efficiency of processes to remove indirect thyroid disrupting chemicals. The TR agonistic and antagonistic activities of water samples after metabolism were investigated by using the TR yeast with the β-galactosidase reporter gene. Neither influent nor effluent increased β-galactosidase expression compared with negative control. However, the influents (sample A) and one effluent (sample B) had TR antagonistic activities that inhibited activity of β-galactosidase expression in a concentration dependent manner (Figure 4). The amiodarone hydrochloride equivalent concentrate ions ranged from 5.21×10^{-4} to 1.75×10^{-3} g/L. Other effluents (sample C, D) were not detected to inhibit activities of β-galactosidase expression, suggesting that the present processes can effectively move the TR antagonists.

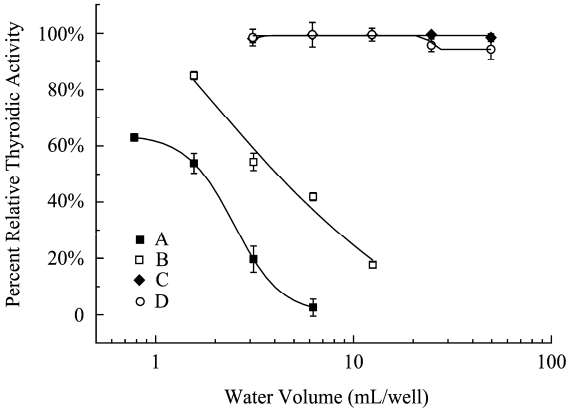

Figure 4. Thyroid receptor (TR) antago nistic activities of water extracts determined by the TR yeast bioassay incorporated with exogenous metabolic activation system (rat liver preparation, S9 mix). The samples were collected from Datansha waste water treatment plant and the sample's antagonistic activity is represented as the percent induction activity relative to the maximum induced by 3,3',5-triiodo-L-thyronine (T_3, 5×10^{-7} mol/L). Values are presented as the average ± standard error (n=3). A=extract of influent, B=extract of effluent after sand filter treatment, C=extract of effluent from train1, D=extract of effluent from train2.

Recent well-designed cohort studies on laboratory animals and *in vitro* research have highlighted several classes of chemicals that particularly likely exert adverse effects on the human thyroid axis[37]. For example, PCBs and brominated flame-retardants are characteristic disruptors of thyroid hormone homeostasis, especially through interaction directly with TR. Li[14] reported that 5×10^{-7} g/L PCBs, poly-brominated biphenyls, and poly-brominated diphenyl ethers after metabolism suppressed TR-mediated transcription in the presence of T_3 and also revealed that the concentrations of these chemicals required for suppressing T_3-induced reporter gene varied considerably, depending on whether the incubation conditions favored metabolism (i.e., in the presence of S9 fraction) or not. One of the prerequisites for PCBs action on the TR, proposed by Lans[38], is hydroxylation at the para-position with one, but preferably two, adjacent halogen substituent. This may explain why the TR antagonist potency of PCBs metabolite is higher than that of nonhydroxylated PCBs. In this work, for influent sample A, the equivalent values of amiodarone hydrochloride with metabolism was 1.75×10^{-3} g/L, which was ten times higher than that of amiodarone hydrochloride without metabolism. Because the neurological development of mammals depends on normal thyroid hormone homeostasis and is likely to be disrupted by thyroid disrupting chemicals even at very low concentration, special attention should be given to the thyroid disrupting effects, especially the biological effects after metabolism.

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