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In vitro thyroid disrupting effects of organic extracts from WWTPs in Beijing

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Abstract

It is generally known that there are many endocrine disrupting compounds (EDCs) in the effluents from wastewater treatment plants (WWTPs). Most research has focused on the occurrence of estrogenic or androgenic activities, while ignoring that there are environmental chemicals disrupting thyroid system, which is essential for growth and development in both humans and animals. In the present work, a two-hybrid yeast assay was conducted to evaluate the removal efficiencies of agonistic or antagonistic thyroid receptor (TR) mediated effects in different treatment processes of three WWTPs located in Beijing. We found no TR agonistic, but TR antagonistic activities in all processes from the WWTPs. The TR antagonistic activities in organic extracts of water samples were then calibrated regarding to a known TR-inhibitor, amiodarone hydrochloride (AH). The observed concentration of TR disrupting substances ranged from 2.35×10^{-8} to 6.19×10^{-7} mol/L AH in Gaobeidian WWTP, 3.76×10^{-8} to 8.75×10^{-8} mol/L AH in Lugouqiao WWTP, and 4.80×10^{-9} to 2.55×10^{-8} mol/L AH in Beixiaohe WWTP. Of the three WWTPs, the removal rates were 92.7%, 42.2%, and 23.1% respectively. Industrial sewage may contain more TR disrupting substances compared with domestic sewage. The recipient waters were found to contain considerable concentrations of TR disrupting substances that may cause adverse effects on the exposed organisms.

Key words: recombinant yeast assay; thyroid receptor; wastewater

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Introduction

In recent years, endocrine disrupting chemicals (EDCs) are emerging as being of major concern for water quality (Heberer et al., 2002; Stackelberg et al., 2004), and an increasing number of EDCs in the water have been found to cause adverse effects in wildlife and human (Colborn et al., 1994). Wastewater treatment plant (WWTP) effluents are considered as one of the major sources of EDCs discharged into the aquatic environment (Johnson et al., 2007). However, many conventional treatment processes in the WWTPs are ineffective in completely removing EDCs (Clara et al., 2005). It was reported that the effluents from WWTPs are mainly responsible for the feminization of fish species in the aquatic environment (Jobling et al., 2009).

Most of the studies on the endocrine disrupting effects of WWTPs have focused on estrogenic or androgenic activity (Nelson et al., 2007; Servos et al., 2005), far less attention has been paid to identifying compounds with thyroid disrupting activity in WWTPs. However, thyroid system is also reported to be vulnerable to endocrine-disrupting effects (Boas et al., 2006), and a number of industrial, medical, agricultural chemicals and their by-products released into the environment may have a potency to disrupt the thyroid system (Brucker, 1998).

Thyroid hormones (THs) are strongly involved in regulating growth, energy metabolism and tissue differentiation (Tata, 1993), and either the THs deficiency or excess will result in a wide variety of postnatal and neuropsychological development in humans and animals (Drury et al., 1984). Given that the major mechanism of TH action involves T_3 binding to its nuclear receptors (Helbing et al., 1992), and many environmental contaminants including BPA, pestisides, phthalates, and phenols, which are usually detected in the wastewaters (Sohoni and Sumpter, 1998; Xu et al., 2005) can directly interfering with thyroid receptors (TRs) (Zoeller, 2005), elimination of TR disrupting substances during wastewater treatment process and assessing the disrupting potency are of considerable environmental importance.

Considering large number of chemicals present in the wastewater, target chemical analyses are not always sufficient. *In vitro* bioassay based on the interaction between chemicals and the thyroid receptors can be used to measure the thyroid disruptive activity of a sample, since majority of EDCs are supposed to act directly with their nuclear receptors either by decreasing normal ligand (T_3) binding or by providing additional ligands that may bind to TRs (Molina-Molina et al., 2006).

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In our previous work, Li et al. (2008a) have developed a novel screening method for chemicals with TR agonistic and TR antagonistic properties using a yeast two-hybrid system which mediates the transcription of β-galactosidase *in vitro* in reporter yeasts (Li et al., 2008b), and found chemicals such as polychlorinated biphenyls (PCBs), flame retardants, phthalates, pesticides, and phenols have the agonistic or antagonistic activity with TR. Considering most of these chemicals could be found in the WWTPs, the aim of the study was to evaluate the removal efficiencies of agonistic and antagonistic TR mediated effects in different treatment processes of three WWTPs located in Beijing and their recipient waters using the two-hybrid yeast assay.

1 Materials and methods

1.1 Chemicals

3,3',5-Triiodo-L-thyronine (T_3, 95%), 3,3',5,5'-tetraiodo-L-thyronine (T_4, 95%), dimethyl sulfoxide (DMSO, 99.5%) were purchased from Sigma Chemical (USA). Amiodarone hydrochloride (AH) was obtained from Shanghai Pharmaceutical (China). HPLC grade dichloromethane, hexane, methanol and *tert*-butyl methyl ether were from Fisher Scientific (USA). For all chemicals, stock solutions were prepared in DMSO. Each test extract was diluted in a 1:2 series in DMSO.

1.2 Sample collection and processing

Composit sampling (24 hr) was conducted in May 2007 at Gaobeidian WWTP, Lugouqiao WWTP and Beixiaohe WWTP in Beijing, China. The sampling sites of Gaobeidian WWTP included influent (A1), effluent after secondary clarifier (A2), effluent after coagulate (A3), effluent after sand filter treatment (A4), and a sample from recipient lake (A5). The sampling sites of Lugouqiao WWTP included influent (B1), effluent after secondary clarifier (B2), effluent after membrane filtration (B3), and a sample from the recipient river (B4). The sampling sites of Beixiaohe WWTP included influent (C1), effluent after secondary clarifier (C2), and a sample from the recipient river (C3).

The water samples (4 L) were collected in pre-cleaned amber glass bottles. Appropriate amount of methanol (2 mL/L, *V*/*V*) was added in each sample right after sampling to suppress possible biotic activities. Samples were kept at a low temperature during transporting and stored at 4°C in a cooling room prior to treatment. All samples were treated within 48 hr.

Water samples and procedure blank (Mili-Q water, 18.2 Ω) were filtered with glass fiber filters (0.45 μm, Whatman, Maidstone, England) to remove insoluble materials. After that, 500 mg Oasis HLB cartridges (Waters, USA) were used for solid phase extraction (SPE) according to the manufacturer's directions. The cartridges were forced under vacuum at a flow rate of approximately 6 mL/min, and then kept under vacuum aspiration for 5 min to dry out. After that, the HLB cartridges were washed with 5 mL of hexane/dichloromethane (7/3, *V*/*V*) twice, 5 mL of *tert*-butyl methyl ether twice, 5 mL of dichloromethane/methanol (9/1, *V*/*V*) twice and 5 mL of methanol, respectively at a flow rate of 1 mL/min. The extracts were then combined and filtered by anhydrous sodium sulphate to remove water and evaporated to dryness in a rotary evaporator (R-200, Buchi, France) at 40°C to 2 mL. The dehydrated extract was blown to dryness under gentle nitrogen flow and reconstituted in 400 μL of DMSO and used for bioassay immediately. Finally extracts were stored at –20°C in glass vials.

1.3 β-Galactosidase assay of TR

The bioassays including agonistic activity test and antagonistic activity test were conducted using yeast strain hTR-GRIP1 as described by Li et al. (2008a). All assays were conducted in at least triplicates. Each assay group included the sample, the positive control (T_3 for agonistic or T_3+AH for antagonistic activities), the negative control (DMSO), as well as the procedural blank. Serial dilutions (5 μL) of test samples were combined with 995 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. For determination of agonistic activities, the extracts were tested in the absence of T_3; and of antagonistic activities in presence of 5×10^{-7} mol/L of T_3 which produced a submaximal stimulatory response (Wang et al., 2005).

The β-galactosidase activity was calculated according to the following equations:

$$u = C_S/t \times V \times D \times OD_S \quad (1)$$

$$C_S = 10^{-6} (A_S - A_B) / \varepsilon \times d \quad (2)$$

where, u is the β-galactosidase activity, C_S is the concentration of *o*-nitrophenol in the enzyme assay reaction mix, t is the incubation duration of the enzyme reaction, V is the volume of the test culture, D is the dilution factor, OD_S is the $OD_{600\,nm}$ of test culture, A_S is the $OD_{420\,nm}$ of the enzyme reaction supernatant of the sample, A_B is the $OD_{420\,nm}$ of the enzyme reaction supernatant of the blank, ε is the molar extinction coefficient for *o*-nitrophenol in the enzyme assay reaction mix, and d is the diameter of the cuvette (Gaido et al., 1997; Routledge and Sumpter, 1996).

Two hundred microliters of the test cultures were transferred into each well of the 96-well plate and incubated at 30°C with vigorous orbital shaking (800 r/min) on a titer plate shaker (Heidolph TITRAMAX 1000, Hamburg, Germany) for 2 hr, then the cell density of the culture was measured at 600 nm wavelength (TECAN GENios A-5002, Salzburg, Austria). After that, a 50 μL test culture was transferred to a new 96-well plate. After addition of 120 μL of Z-buffer (g/L, $Na_2HPO_4{\cdot}7H_2O$ 16.1; $NaH_2PO_4{\cdot}H_2O$ 5.5; KCl 0.75; $MgSO_4{\cdot}7H_2O$ 0.246) and 20 μL chloroform, the assays were carefully mixed and preincubated for 10 min at 30°C. Then the enzyme reaction was started by adding 40 μL *o*-nitrophenyl-β-D-galactopyranoside (13.3 mmol/L, dissolved in Z-buffer), then incubated at 30°C on a titer plate shaker for 60

min. The reactions were terminated by the addition of 100 μL Na_2CO_3 (1 mol/L). 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 $OD_{420\ nm}$ was determined.

1.4 Control assays

The control assays of the yeast assay were performed for the procedure blank. All of the induction or inhibition activities of procedure blank were less than 5% and no concentration dependent relationships were found.

To exclude the false results caused by cytotoxicity, the cell viability was determined spectrophotometrically as a change of cell density ($OD_{600\ nm}$) in the assay medium. All the tests were conducted along with the determination of the cell viability. The procedural blank was tested in the same concentration to monitor for a false-positive result. The detailed steps had been described by Li et al. (2008a).

2 Results and discussion

No TR agonistic activities were observed in the extracts even when the water samples were 25 times concentrated (Fig. 1a). However, all samples had TR antagonistic activities that inhibited activity of β-galactosidase expression in presence of submaximal T_3 concentration (5×10^{-7} mol/L). The concentration dependent curves of TR antagonistic activities are shown in Fig. 1b.

The TR antagonistic activities of water samples were then calibrated regarding to a known TR-inhibitor, amiodarone hydrochloride (AH) (Table 1). The concentration dependent relationship was reported by Li et al. (2008a).

Table 1 Two-hybrid TR bioassay used to determine the possible endocrine disrupting potency

Reference material	Endpoint	REC50 or RIC50* (mol/L)
T3	Agonistic activity through TR	1.1×10^{-7}
T4	Agonistic activity through TR	2.7×10^{-7}
AH	Antagonitic activity through TR in the presence of T_3	2.4×10^{-7}

TR: thyroid receptor; T_3: 3,3',5-triiodo-L-thyronine; T_4: 3,3',5,5'-tetraiodo-L-thyronine; AH: amiodarone hydrochloride; REC50: the concentration inducing 50% of the maximum effect; RIC50: the concentration causing a 50% inhibition of the maximum effect. * Li et al., 2008.

The observed concentration of TR disrupting substances ranged from 2.35×10^{-8} to 6.19×10^{-7} mol/L AH in Gaobeidian WWTP, 3.76×10^{-8} to 8.75×10^{-8} mol/L AH in Lugouqiao WWTP, and 4.80×10^{-9} to 2.55×10^{-8} mol/L AH in Beixiaohe WWTP (Table 2).

The Gaobeidian WWTP received industrial and domestic sewages, while the other two received mostly domestic sewage. In Gaobeidian WWTP, secondary clarifier, coagulation, coal and sand filtration processes could totally remove 92.7% of TR disrupting substances compared with the original water. In Lugouqiao WWTP, secondary clarifier and membrane filtration processes can totally remove 42.2% of TR disrupting substances compared with the original water. In Beixiaohe WWTP, secondary clarifier can remove 23.1% of TR disrupting substances compared with the original water. However, the recipient waters were also found to have considerable concentration of TR disrupting substances even after dilution and degradation in the environment. The higher removal efficiency of TR disrupting substances in Gaobeidian WWTP may be due

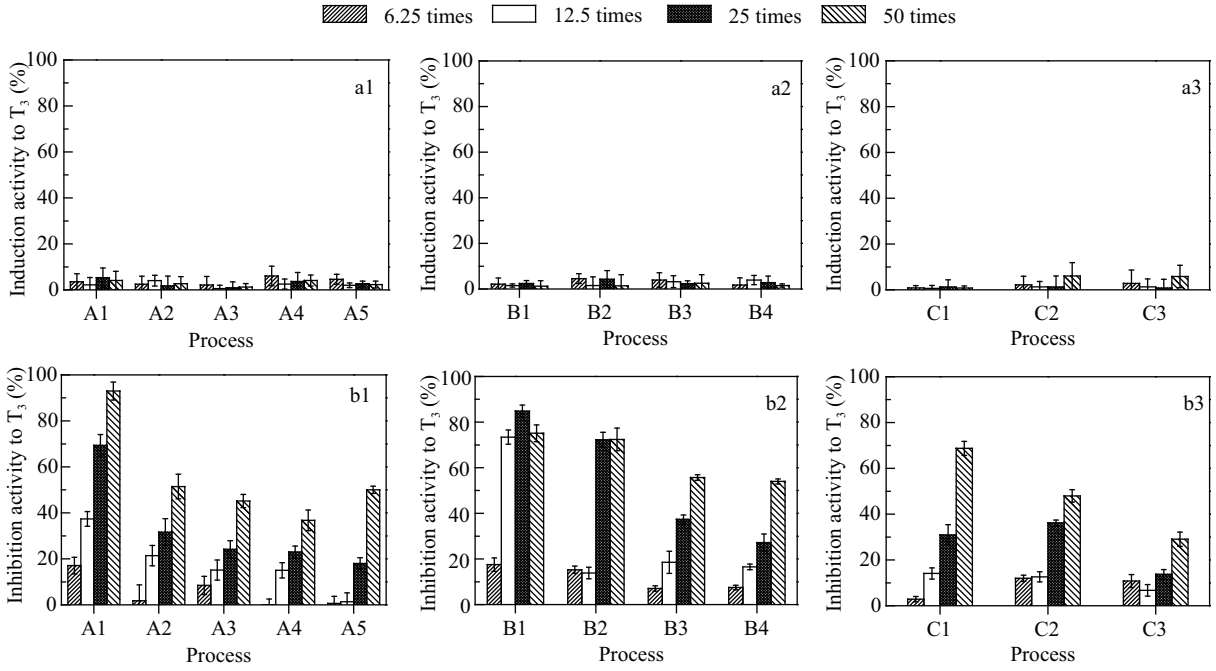

Fig. 1 Thyroid receptor (TR) agonistic activities (a) and TR antagonistic activities (b) of water extracts of Gaobeidian (A), Lugouqiao (B), and Beixiaohe (C) wastewater treatment plant determined by the TR yeast bioassay. The sample's agonistic or antagonistic activity is represented as the percent 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). A1, B1, C1: influent; A2, B2, C2: effluent after secondary clarifier effluent; A3: effluent after coagulate; B3: effluent after membrane filtration; A4: effluent after sand filter treatment; A5, B4, C3: recipient lake. 6.25, 12.5, 25, and 50 times denote the concentration folds of the original water.

Table 2 Toxic equivalent of the effluents from three WWTPs of two-hybrid TR bioassay

	Process	TR agonistic activity (mol T_3-EQ/L)	TR antagonistic activity (mol AH-EQ/L)
Gaobeidian WWTP	Influent (A1)	ND	6.19×10^{-7}
	Secondary clarifier (A2)	ND	1.40×10^{-7}
	Coagulate (A3)	ND	5.41×10^{-8}
	Sand filter treatment (A4)	ND	4.50×10^{-8}
	Recipient lake (A5)	ND	2.35×10^{-8}
Lugouqiao WWTP	Influent (B1)	ND	8.75×10^{-8}
	Secondary clarifier (B2)	ND	6.17×10^{-8}
	Membrane filtration (B3)	ND	5.06×10^{-8}
	Recipient river (B4)	ND	3.76×10^{-8}
Beixiaohe WWTP	Influent (C1)	ND	2.55×10^{-8}
	Secondary clarifier (C2)	ND	1.96×10^{-8}
	Recipient river (C3)	ND	4.80×10^{-9}

ND: not detection.

to the higher concentration of TR disrupting substances in the influent, which is about 10 times higher than those from Lugouqiao and Beixiaohe WWTPs. Considering that even transient disruption of normal THs will lead to disastrous outcomes (Haddow et al., 1999) and trace levels of EDCs are discovered in water associated with WWTP effluents (Routledge et al., 1998), WWTP effluents contain TR disruptors may have complex adverse effects on exposed organisms.

In the present study it showed TR antagonists in the WWTP effluents. There were few reports on TR antagonists in the wastewater samples. Murata and Yamauchi (2008) detected strong TR agonistic activity, but not TR antagonistic activity, in the dichloromethane and dichloromethane/methanol fractions of effluents from the sewage treatment plant (STP) in Japan. Gutleb et al. (2005) reported that sediment extracts showed TR antagonist activities using T-screen method and they proposed that it was partly related to the wastewater discharges. Our results support their evidences that there are a large amount of TR antagonists in wastewater and a part of them will be discharged into the recipient water. There were TR disrupting activities in other environmental samples. Ishihara et al. (2009) found strong TR antagonistic activities in water samples from paper manufacturing plants (PMPs) and found TR antagonistic activities in waters of the Khlong U-Taphao at every location sampled using TR-mediated luciferase gene activation.

Of the TR antagonists in wastewater, some phthalates including dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP), diethy phthalate (DEP) and benzyl butyl phthalate (BBP) were detected (Zhou and Wang, 2009). Phthalates are synthetic compounds widely used as polymer additives in plastics to improve their flexibility (Sharman et al., 1994). Many phthalates e.g., DBP and DEHP can affect the thyroid system in laboratory animals (Hinton et al., 1986; Howarth et al., 2001) or have antagonistic acitivities to TR *in vitro* (Sugiyama et al., 2005). Due to the widespread application of phthalates, they have been widespread pollution in China (Cai et al., 2007). Therefore, phthalates may contribute to a part of the TR antagonistic activities in the wastewater. Other chemicals such as PCBs, flame retardants detected in the wastewater (van der Oost et al., 2003; Vega-Lopez et al., 2007), may also contribute to the TR agonistic or TR antagonistic activities.

3 Conclusions

The results presented in this article suggested that the wastewater and the receiving water in Beijing contain xenobiotics that can bind to TR and manifest TR antagonistic effects. The processes are not able to remove all TR antagonists and the recipient waters were found to contain considerable concentration of TR disrupting substances. Good technologies are needed to reducing TR disrupting pollutants in the WWTPs and it is important to monitor TR disruptors in the WWTP effluents for water quality safety. The two-hybrid yeast assay can be used as a fast screening tool for identification of environmental samples that may induce functional agonistic and antagonistic TR-mediated effects.

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