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EVALUATION OF ESTROGENICITY OF SEWAGE EFFLUENT AND RECLAIMED WATER USING VITELLOGENIN AS A BIOMARKER

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Abstract—To evaluate the quality of reclaimed water, the estrogenicity of effluent from a sewage treatment plant and of reclaimed water treated with coagulation–sedimentation–filtration using the effluent as raw water was assessed using vitellogenin (VTG) as a biomarker. After a three-week exposure, significant ($p < 0.05$) induction of VTG occurred in female crucian carp (*Carassius carassius*) exposed continuously to the secondary effluent and reclaimed water with different dilutions (12.5, 25, 50, and 100% for secondary effluent; 50 and 100% for reclaimed water); no induction of VTG was detected when exposed to 12.5 and 25% reclaimed water. For male fish, however, only 100% secondary effluent induced the production of VTG (mean ± standard deviation, 38.6 ± 9.8 μg/ml). When the exposure time was prolonged to three months, VTG was induced significantly in both females and males at all gradient concentrations of secondary effluent and at 50 and 100% reclaimed water. The results indicated that no obvious VTG was detected in fish exposed to reclaimed water diluted more than fourfold. Ozonation of the secondary effluent under an ozone consumption dose of 8.5 mg/L resulted in a VTG level equal to that of 12.5% secondary effluent or 50% reclaimed water. Furthermore, VTG induction reflects the cumulative effects of estrogenic activity in the secondary effluent and reclaimed water compared with the in vitro assays, in which estrogenic activities in effluent changed markedly during the experiment.

Keywords—Estrogenicity Vitellogenin Crucian carp Secondary effluents Reclaimed water

INTRODUCTION

Reclaimed wastewater is the effluents of a sewage treatment plant (STP) that are adequately and reliably treated with different wastewater treatment process and is an important supplemental water resource for urban use, food crop irrigation, and recreational impoundments in many countries, especially for drought areas [1,2]. Besides the sensory problems related to odor, color, and turbidity, the occurrence of endocrine-disrupting chemicals (EDCs) in the reclaimed water is of concern when the water is used for agricultural irrigation, landscape irrigation, and groundwater recharge.

Earlier laboratory and field data showed that most STPs with conventional activated sludge processes cannot completely eliminate EDCs from sewage [3,4]. The residual EDCs in the secondary effluents were considered to be related to some reproductive disturbances in wild fish, such as the induction of the female-specific protein vitellogenin (VTG) [5–7], alteration of serum sex steroid level [8], high prevalence of intersex gonads [9,10], and reduced testis size [7]. These abnormal phenomena indicate that further treatment is required when the secondary effluent is used for environmental supplementation [11–13].

The coagulation–sedimentation–sand filtration process has been widely used for reclamation of the secondary effluents from STPs [14,15] because of its low cost and high performance in removing colloidal particles and turbidity from wastewater [12,13]. Coagulation using polyaluminum chloride (PAC) as the coagulant was reportedly able to remove from 30 to 82% of natural and synthetic estrogens from secondary effluents [16]. It is not clear, however, if the conventional reclamation treatment is sufficient to ensure the ecological safety of the reclaimed water. In recent years, several studies have shown that ozone (O_3) could oxidize estrogens, such as 17β-estradiol, estrone, and 17α-ethinylestradiol, in drinking water and wastewater either by ozone directly or by hydroxyl radicals (•OH) [17–20], and it has been regarded as a very promising technology for reclamation of effluent [15,21].

Compared to single-chemical analyses and in vitro assays, in vivo assays are believed to provide a direct assessment of the overall environmental impacts of water. In the present study, VTG analysis was used to evaluate the estrogenic activities of secondary effluent and reclaimed water from the Gaobeidian STP (G-STP) in Beijing (China). Crucian carp (*Carassius carassius*), a common Chinese species, was exposed to the secondary effluent and reclaimed water treated with the conventional process, and the plasma VTG level was analyzed using an enzyme-linked immunosorbent assay [22]. For comparison, the estrogenic activities of the secondary effluent and reclaimed water also were evaluated using the recombinant yeast assay.

MATERIALS AND METHODS

Sample collection

With its capacity of 100 million tons/d, the G-STP is the largest STP in China. It uses two processes: The anoxic–anaerobic–aerobic process, and the anoxic–aerobic process. Part of the secondary effluent was reclaimed following successive treatment with coagulation (15 mg Al_2O_3/L PAC), sedimentation, and sand filtration. For comparison, the secondary effluent also was treated with ozone under an ozone consumption dose of 8.5 mg/L. The secondary effluent, reclaimed water,

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and ozonated secondary effluent were selected for fish cultures during the exposure experiments.

Estrogenicity in secondary effluent and reclaimed water

The yeast two-hybrid assay system with estrogen receptor α and coactivator TIF2 (transcriptional intermediate factor 2) was used to investigate the transcriptional activation induced by xenoestrogens in the G-STP effluent. This method has been described in detail previously [23].

Using flow-proportional samplers at the rate of 100 ml/h (6712R; ISCO, Lincoln, NE, USA), 24-h composite samples were collected each day for six weeks. The daily 24-h composites were stored at 4°C, and then two-week composites were pooled. Thus, three samples of secondary effluent and reclaimed water were collected during the experiment (i.e., the first two weeks, second two weeks, and third two weeks). Next, 2-L composites every two weeks were filtered through glass microfiber filters (pore size, 0.45 μm; Whatman, Maidstone, Kent, UK) after the pH was adjusted to 2 to 3 with hydrochloric acid. The samples were then percolated through a C18 solid-phase extraction cartridge (Waters, Milford, MA, USA) conditioned with 10 ml of methanol and 10 ml of deionized water, and the cartridges were dried under a nitrogen stream. Elution was carried out with 5 ml of hexane and 5 ml of dichloromethane. The combined extracts were dried under a nitrogen stream and then dissolved in 100 μl of dimethyl sulfoxide. The estrogenic activity of the samples was determined by directly comparing the concentration–response curves of the individual effluent samples with the concentration–response curves obtained for reference standard chemicals (i.e., 17β-estradiol). The amount of estrogenic activity is presented as ng/17β-estradiol equivalents (EEQs).

Short-term exposure to secondary effluent and reclaimed water

Nine 100-L glass tanks were supplied with a series of secondary effluent and reclaimed water dilutions produced by mixing with different percentages of activated carbon–dechlorinated tap water. Nominal concentrations of the secondary effluent and reclaimed water were 100, 50, 25, and 12.5%, and dechlorinated tap water was used as control. The flow rate through each tank was 1.2 L/min. At the beginning of the experiment, each tank contained 100 juvenile (age, three months) crucian carp (weight, 16.7 ± 3.2 g; length, 9.8 ± 2.5 cm [both mean ± standard deviation]). All fish were fed with frozen shrimp twice daily throughout the trial (5% body wt/d). The tanks were aerated to ensure sufficient oxygen supply, and the water temperature was maintained at 22 ± 1°C (mean ± standard deviation) in all tanks.

After one-, two-, and three-week exposures, male (n = 6–8 per group) and female (n = 10–12 per group) crucian carp were sampled from different groups. After measuring the weight and length of the fish, approximately 0.2 ml of blood was extracted from the caudal vein by means of heparinized syringes. Blood samples were centrifuged at 10,000 rpm for 5 min, and the plasma samples mixed with aprotinin (1 Trypsin Inhibitory Unit/ml) were frozen at −80°C until analysis. The gonads were weighed, and the gonadosomatic index (GSI = [gonad weight/body wt]·100%) was calculated.

Effect of secondary effluent, reclaimed water, and ozonated secondary effluent with long-term exposure

Considering that the short-term exposure is not representative of the lifelong exposure that occurs in wild fish and the need for a long-term trial to determine the ecological impacts of reclaimed effluents on wildlife [24,25], juvenile crucian carp in the present study was exposed to secondary effluent, reclaimed water, and ozonated secondary effluent for three months to evaluate the potential long-term health and ecological risk hazards of effluents. Ten 100-L glass tanks were supplied with a series of secondary effluent (100, 50, 25, and 12.5%), reclaimed water (100, 50, 25, and 12.5%), and 100% secondary effluent treated with ozone. Fifty juvenile crucian carp were employed in this system, and other conditions were similar to those of the short-term exposure system. After the three-month exposure, males (n = 6–8) and females (n = 10–12) were sampled as described above.

VTG determination

Blood plasma was assayed for VTG concentration using the previously validated, sandwich enzyme-linked immunosorbent assay [22]. Briefly, the polyclonal antibodies against crucian carp VTG (c-VTG) were coated on a 96-well microtiter plate for a night at 4°C. Purified c-VTG or diluted samples were incubated for 1 h at 37°C. Then, after the monoclonal antibodies against c-VTG were added, the plate was again incubated for 1 h at 37°C. Next, the plate was incubated yet again for 1 h at 37°C following the addition of 100 μl of the goat anti-mouse IgG horseradish peroxidase (Sigma, Louis, MO, USA). The TMB (3,3′,5,5′-tetramethylbenzidine) substrate was added at 100 μl/well after the plate had been washed five times with PBST (10 mM phosphate-buffered saline and 0.05% Tween 20; Sigma-Aldrich, Louis, MO, USA). The color development was stopped after 15 to 25 min in the dark by adding 50 μl of 2 M H_2SO_4 to each well. The plate was read at 450 nm. The VTG concentrations were calculated according to the standard curve, which was established using the results of purified c-VTG.

Statistical analysis

We performed the statistical analysis using SPSS® software (Ver 11.0; SPSS, Chicago, IL, USA). Plasma VTG and GSI values were analyzed using one-way analysis of variance to compare differences among fish exposed to the secondary effluent, reclaimed water, or secondary effluent treated with ozone versus control. Significant differences are accepted at $p < 0.05$ for all comparisons. All data are expressed as the mean ± standard deviation.

RESULTS AND DISCUSSION

In vitro assay of estrogenic activities

The samples were concentrated by solid-phase extraction. Then, the extracts were serially diluted, and the estrogenicity at different dilutions was detected using the recombinant yeast assay. Figure 1 shows the dose–response curves of individual effluent samples together with that of 17β-estradiol. Among the six samples, including three secondary effluent samples and three reclaimed water samples, only one effluent and one reclaimed water sample collected during the second two weeks (secondary II and reclaimed II) showed a clear dose–response effect (Fig. 1b): For the secondary effluent, 40.8 ± 13.6 ng EEQ/L, and for the reclaimed water, 16.8 ± 10.8 ng EEQ/L. Approximately 60% of the estrogenic activity in the secondary effluent was removed by the conventional reclamation treatment. The other two secondary effluent and two reclaimed water samples collected during the first two weeks and the third two weeks did not show perceptible estrogenic activity,

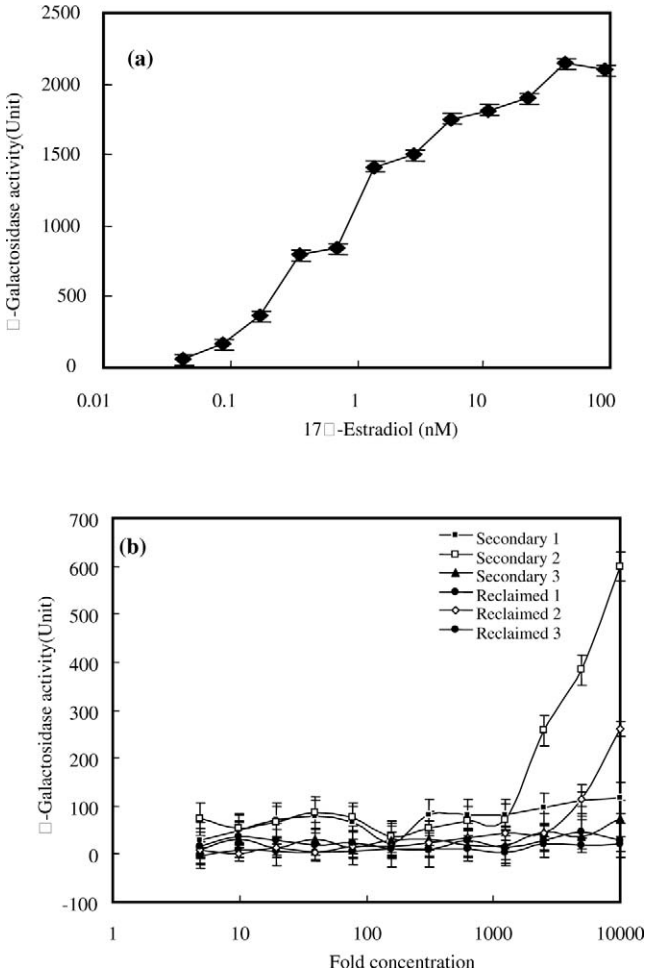

Fig. 1. Estrogenic activities in secondary effluent and reclaimed water collected from Gaobeidian Sewage Treatment Plant (G-STP). (**a**) Dose–response curve of 17β-estradiol standard. (**b**) Dose–response curve of individual effluent samples. All data was expressed as the mean ± standard deviation. 1 = first two weeks; 2 = second two weeks; 3 = third two weeks.

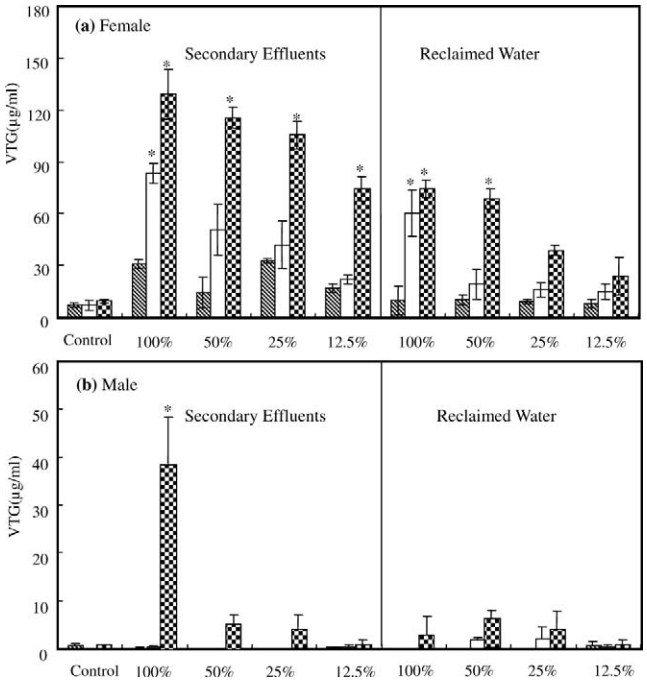

Fig. 2. Vitellogenin (VTG) concentration (μg/ml; mean ± standard error) in juvenile (**a**) female and (**b**) male crucian carp (*Carassius carassius*) exposed to various concentrations of secondary effluent and reclaimed water. *$p < 0.05$. ▨ = one week; □ = two weeks; ▩ = three weeks.

and all had levels of less than the detection limit (4.2 ng EEQ/L) with this assay. These results showed the great variability in estrogenicity among effluent samples, and such phenomena also have been reported previously [26]. Although we cannot reasonably explain the great variability in estrogenicity among effluents, such variability suggests that an in vivo assay is necessary to directly assess the estrogenicity of effluent, because an in vivo assay can detect the cumulative estrogenic effects of the effluents during the test.

VTG and GSI responses after one-, two-, and three-week exposures

Figure 2 shows the variations of plasma VTG concentration in both females and males with exposure time and the nominal concentrations of the secondary effluent and reclaimed water. For males and females exposed to tap water (control), the VTG concentrations were 0.4 to 0.6 and 7.1 to 9.3 μg/ml, respectively, over the three-week exposure, indicating that plasma VTG was not induced in tap water. For females exposed to the secondary effluent, however, significant induction of VTG was observed in all dilutions (100, 50, 25, and 12.5%), as shown in Figure 2a. The VTG level increased with increasing exposure time. The 100% secondary effluent induced a significant ($p < 0.05$) 12- and 13-fold increase in plasma VTG concentration after a two- and a three-week exposure, respectively. The 12.5, 25, and 50% secondary effluents also induced significant ($p < 0.05$) VTG production after a three-week exposure. A significant ($p < 0.05$) concentration-related response was observed for plasma VTG induction with increasing concentrations of the secondary effluent for three-week exposure. For the males, on the other hand, only those exposed to 100% secondary effluent for three weeks significantly ($p < 0.05$) induced VTG production (Fig. 2b). These results indicated that the VTG response in females was more sensitive than that in males, a phenomenon that has been reported for immature rockfish (*Sebastes schlegeli*) after injection of 4-nonylphenol [27] and immature rainbow trout (*Oncorhynchus mykiss*) exposed to effluents for approximately 10 d [26], suggesting differences in uptake or processing of chemicals from the aquatic environment in males and females. Moreover, the different estrogenic response (VTG induction) might be related to the variations in the estrogen receptor response between males and females after exposure to estrogens [27]. So, from the viewpoint of the sensitivity of the VTG response to the estrogenicity of water, juvenile femal crucian carp are suitable experimental animals.

As for the reclaimed water, only the 100 and 50% groups showed significantly induced VTG in females after a three-week exposure, and no VTG was detected in males exposed to different gradients of reclaimed water. This suggests that the estrogenicity was decreased markedly by the coagulation treatment.

VTG and GSI responses after a three-month exposure

Figure 3 shows VTG induction in fish after a three-month exposure to the secondary effluent, reclaimed water, and ozonated secondary effluent. A higher level of VTG was measured

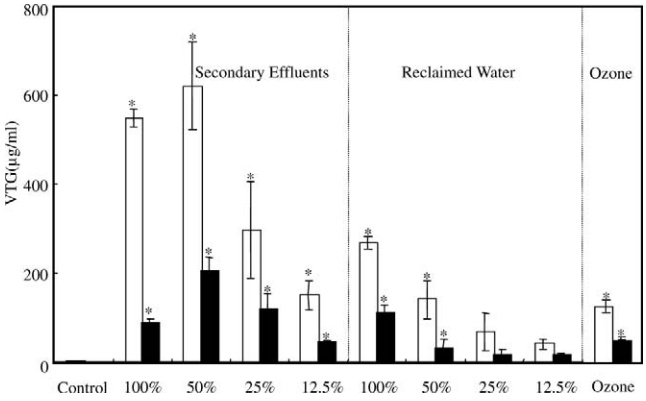

Fig. 3. Vitellogenin (VTG) concentration (μg/ml; mean ± standard error) in juvenile female (□) and male (■) crucian carp (*Carassius carassius*) exposed to various concentrations of secondary effluent, reclaimed water, and secondary effluent treated with ozonation for three months. $*p < 0.05$.

in all males and females compared with levels in a three-week exposure. Compared with the control values (females, 2.6 ± 0.3 μg/ml; males, 0.6 ± 0.1 μg/ml), VTG concentrations in both females and males increased markedly ($p < 0.05$) with an increasing proportion of secondary effluent from 12.5 to 50% and then decreased at 100%. In addition, the GSI suppression was found in males exposed to the 100% secondary effluent (Fig. 4), suggesting that an adverse effect on gonadal development occurred. This phenomenon also has been reported in fish that live in rivers contaminated by sewage effluent [6,28]. It should be noted that VTG induction occurred in all males after the three-month exposure to the secondary effluent, indicating that estrogenic responses in wild fish cannot necessarily be predicted from short-term exposure. Thus, a longer-term exposure assay was necessary to assess the estrogenicity of effluent.

As for fish exposed to the 50 and 100% reclaimed water, VTG concentrations increased markedly ($p < 0.05$) not only in females (140.9 ± 43.4 and 269.0 ± 15.3 μg/ml, respectively) but also in males (30.7 ± 5.7 and 111.2 ± 17.5 μg/ml, respectively), suggesting the existence of a long-term exposure risk even for males. The VTG levels were significantly lower, however, than the levels in those exposed to the corresponding secondary effluent, indicating the relative effectiveness of the conventional reclamation treatment for removing the estrogenic activities. On the other hand, no significant ($p > 0.05$) VTG induction was found in both females and

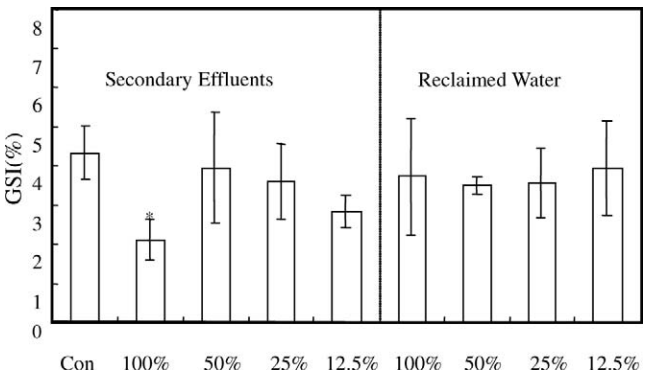

Fig. 4. Changes of the gonadosomatic index (GSI) of juvenile male crucian carp (*Carassius carassius*) exposed to secondary effluent and reclaimed water for three months. $*p < 0.05$.

males when exposed to the 12.5 and 25% reclaimed water. These results suggest that a dilution factor of four would be necessary for reuse of the reclaimed water.

For cities like Beijing, which do not have much dilution water, it is necessary to remove the EDCs more effectively. So, the efficiency of ozonation for diminishing the VTG induction was evaluated briefly in the present study. The VTG levels in fish exposed to the ozonated secondary effluent were 114.6 ± 9.3 μg/ml for females and 49.6 ± 2.9 μg/ml for males, which corresponded to levels induced by 12.5% secondary effluent or 50% reclaimed water. Therefore, a combination of conventional reclamation treatment and ozonation might be a sound strategy from the viewpoint of complete removal of the estrogenic activities from the secondary effluent.

In the present study, an in vitro assay using recombinant yeast and an in vivo assay using VTG analysis were employed to evaluate the estrogenic activities in the secondary effluent and reclaimed water. The recombinant yeast assay is a useful tool for the first-level screening of estrogenic activity because of its rapidity, simplicity, and high analytical throughput. As shown in Figure 1b, however, the estrogenic activity in the secondary effluent could not be detected in some cases, because the results did not reflect the cumulative estrogenic effects of the effluents. In comparison, the in vivo VTG bioassay, especially with a relatively long exposure time, demonstrated the cumulative estrogenic effects in water as well as the removal effects by different treatment strategies. Thus, the in vivo tools are better for evaluating the ecological effects of wastewater discharging and reuse of reclaimed water.

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