

## Enhanced Vitellogenin Induction of Secondary Effluents by Chlorination

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Previous work has demonstrated the presence of both male and female gonad characteristics within the same fish (*Rutilus rutilus*). These fish were found inhabiting freshwater near treated wastewater discharges (Jobling et al. 1998). This phenomenon has been linked with both domestic and municipal sewage effluents. It was reported that dose- and time-dependent induction of plasma vitellogenin (VTG, the precursor of yolk protein) occurred in caged fish exposed to municipal sewage effluents, and the persistent feminization of the reproductive ducts in presumed male fish has been found (Rodgers-Gray et al. 2000; 2001). These studies mainly focused on evaluating estrogenic activity in municipal sewage effluents treated with conventional processes. Although chlorination is often used in treatment processes in sewage treatment plants (STPs), the effects of chlorination on the estrogenic activity of secondary effluents are unclear.

In this study, zebrafish (*Danio rerio*) VTG (zf - VTG) and medaka early - life development were used to monitor the effects of estrogenic activity in chlorinated secondary effluent from Fangzhuang STP in Beijing, which was compared with that in secondary effluent. Eleven polycyclic aromatic hydrocarbons (PAHs), from the 16 PAHs suggested by the US EPA as the principal contaminants in effluents before and after chlorination, were also analyzed.

### MATERIALS AND METHODS

The standards of PAHs (naphthalene, acenaphthene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, and dibenzo(a,h)anthracene) were purchased from SUPELCO (USA). Hexane and dichloromethane were HPLC grade.

The secondary effluents after conventional treatment processes in Fangzhuang STP were transported to the lab every day. First, the effluent was chlorinated with 5 mg/L NaOCl for two hours. Then, the surplus chlorine was removed by adding

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sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_4$ ), and the pH was adjusted to  $7.8 \pm 0.2$ . Tap water filtered through activated carbon was used as the control. A laboratory-constructed flow-through system was employed. Each unit of the system was composed of two chambers with a volume of 40 liters for exposure and 100 liters for cycling of the effluents. Twenty mature male zebrafish (length =  $3.50 \pm 0.50$  cm; weight =  $0.25 \pm 0.05$  g) were exposed to secondary effluent and chlorinated secondary effluent, of which 40 % was changed every day. The fish were fed twice with commercial pellets (less than 5 % of body weight/day) every day and the remaining food was removed after one hour. The temperature was controlled at  $23 \pm 1$  °C, and dissolved oxygen content saturation was kept above 4.0 mg/L. The photoperiod was automatically maintained at 14 hr (light): 10 hr (dark). After 2 weeks, the zebrafish were frozen in liquid nitrogen in order to analyze VTG.

The whole body was homogenized in buffer (10 mM Tris-HCl containing 0.9 % NaCl, 0.05 % Tween 20, 1 % BSA, 0.1 % aprotinin, pH = 7.4) at a ratio of 1:2 (w/v). After centrifugation of the homogenates (12,000 rpm, 30 min, 4 °C), the supernatant was stored at -80 °C. Sandwich ELISA kits (Bio-sense, Norway) were used to determine the VTG concentration (Holbech et al. 2001). Briefly, a 96 - well microplate was coated at 37 °C for 2 hr with polyclonal antibody against zf - VTG [100  $\mu\text{l}$ /well, dilution with carbonate buffer (CBS, 0.05 M, pH = 9.6)]. After washing three times with PBST buffer [0.05 % Tween 20 in PBS (0.01 M, pH = 7.6)], the plate was blocked with BSA (1 %) for 2 hr at 37 °C. After washing with PBST buffer three times, the samples were added into wells, and incubated at 37 °C for 1 hr. Then, the plate was incubated at 37 °C for 1 hr with monoclonal antibody and subsequently with goat anti-mouse IgG-horseradish peroxidase (Zhongshan, Beijing). The plate was washed, and then 100  $\mu\text{l}$ /well freshly prepared substrate [3 mg/ml of *p*-nitrophenyl phosphate (Sigma, 104-105, USA) in substrate buffer (100 mM sodium phosphate, 50 mM sodium citrate, 0.05 %  $\text{H}_2\text{O}_2$ ; pH = 5.0)] was added. The color reaction was stopped after about 30 min in the dark by adding 50  $\mu\text{l}$   $\text{H}_2\text{SO}_4$  (2 M). Absorbance (OD) was read at 490 nm with a microplate reader (Bio-rad 550). The working range of the VTG standard curve was from 0.25 to 31.3 ng/ml.

In order to determine possible impacts of EDCs in effluents during early-life development, the larval medaka (*Oryzias latipes*) 30-day post hatch was exposed to the effluents before and after chlorination. In brief, 40 larvae were exposed to effluents before and after chlorination in a 40-liter chamber, carried out with a similar laboratory-constructed flow-through system as described above. Each chamber was divided into two with a mesh partition. The larvae from each group were divided into two subgroups and placed into two chambers, that is, 10 larvae and the other 30 larvae were apart in the same exposure chamber. One subgroup (10 larvae) was monitored after exposure 1, 2, 3 and 4 weeks. This was being

monitored continuously by measuring the lengths and weights. All of the larvae were fed twice with hatched shrimp freshly (INVE, USA).

500 - ml effluent samples were collected daily, and stored at 4 °C. After two weeks, all samples were mixed thoroughly and two liters were used for analyzing PAHs. After the samples were first filtered with 0.45 µm glass fiber filter paper (Whatman) and the pH was adjusted to 2 - 3, each sample was percolated through a C18 solid phase extraction cartridge (Waters, USA), and then 5 ml hexane and 5 ml dichloromethane were used to elute the cartridge. The eluate was dried under a gentle stream of nitrogen, and then dissolved in 100 µl of hexane. The mass spectrometer (Q-Mass-910, HP) was operated in the electron impact ionization mode with an ionizing energy of 70 eV. The injector temperature was maintained at 280 °C, and the transmission line temperature was kept at 260 °C. An HP - 5MS capillary column was programmed to increase from 40 °C to 280 °C at 10 °C/min (keeping this temperature for 10 min). Injection was performed in the splitless mode and helium was used as the carrier gas. 2 - µl samples were injected manually. The detection limits and recoveries of PAHs were listed in Table 1.

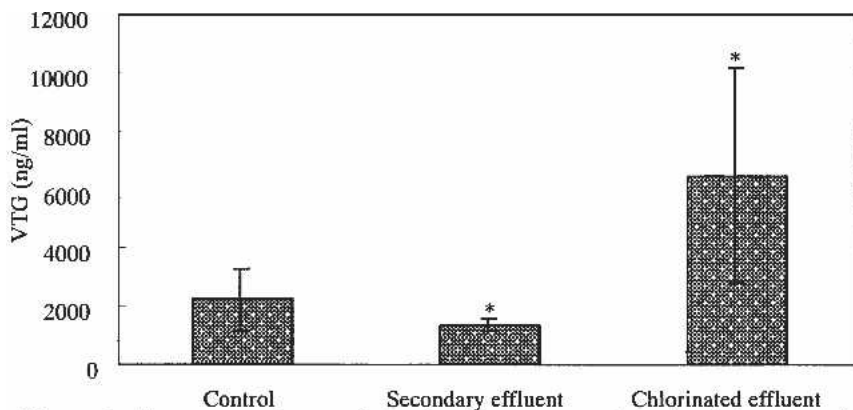
**Table 1.** Recoveries and detections limit of PAHs

PAHs	Detection limit (ng/L)	Recovery (%)
naphthalene	0.33	69.2
acenaphthene	0.17	117.7
phenanthrene	0.42	119.8
anthracene	0.33	62.8
fluoranthene	0.25	104.4
pyrene	0.13	120.9
benzo(a)anthracene	0.13	81.3
chrysene	0.13	92.3
benzo(a)pyrene	2.00	76.8
indeno(1,2,3-cd)pyrene	1.25	113.5
dibenzo(a,h)anthracene	3.33	95.2

One-way analysis of variance (ANOVA) was used to determine differences in VTG induction and growth among experimental groups. Differences were considered significant if  $p < 0.05$ .

## RESULTS AND DISCUSSION

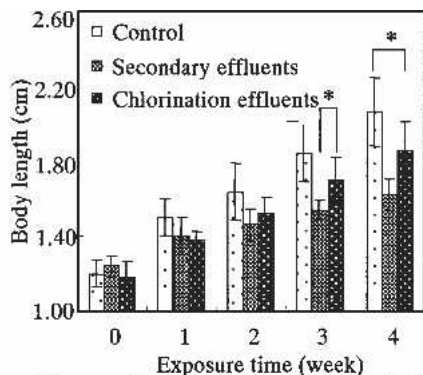
As shown in Figure 1, VTG concentrations in secondary effluents were  $1362.1 \pm 221.7$  ng / ml, which was significantly lower than that in the control ( $2236.1 \pm$



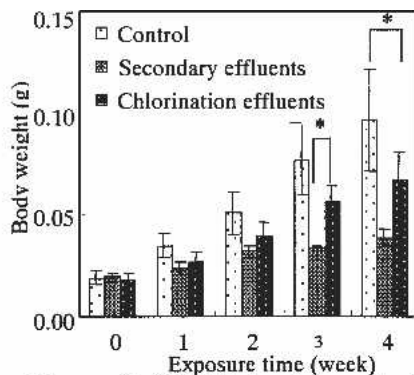
**Figure 1.** Vtg suppression or induction in mature male zebrafish exposed to secondary effluent or chlorinated effluent. Asterisk indicates that the Vtg concentrations in zebrafish exposed to effluents changed significantly compared with that in the control ( $p < 0.05$ ). \*,  $p < 0.05$ .

1067.6 ng/ml) ( $p < 0.05$ ). This phenomenon was similar to the results reported by COMPREHEND (Pickering and Sumpter, 2003); that is, some effluents did not induce VTG *in vivo* even if significant estrogenic activities were detected *in vitro*. To better understand this issue, some PAHs suggested by the US EPA as principal contaminants of effluents were analyzed. Antiestrogenicities for these agents were reported by Arcaro et al. (1999). As a result, five PAHs, acenaphthene, phenanthrene, anthracene, fluoranthene, and dibenzo(a,h)anthracene, were detected and the total concentration was 887 ng/L in secondary effluents (Table 2). Such a concentration level would induce limited antiestrogenicity according to the results reported by Engwall et al. (1997). At the same time, we found that the larvae lengths and weights in secondary effluents were obviously lower than that in the control group after two weeks exposure ( $p < 0.05$ , Figures 2 and 3), suggesting that the secondary effluent had a negative effect on normal physiological metabolism, which would result in a lack of significant VTG induction, in contrast to the control.

On the other hand, the VTG concentration in chlorinated effluents was  $6481.8 \pm 3682.5$  ng/ml, which is about three times and four times higher than those in the control and in the secondary effluent ( $p < 0.05$ ), respectively (Figure 1). That is, from the view of VTG induction, the estrogenicity of chlorinated effluent became obvious. In fact, some studies have found that several endocrine disrupting chemicals (EDCs) such as 17 $\beta$  - estradiol (E2) and bisphenol A (BPA) can be reactive with NaOCl during the chlorination process, and their by - products still showed estrogenic activity after chlorination based on both *in vivo* and *in vitro* studies (Hu et al. 2002; Tabata et al. 2003; Alum et al. 2004). Therefore, the



**Figure 2.** Length development of larval medaka exposed to the secondary effluents before and after chlorination for four weeks (n = 10). \*,  $p < 0.05$



**Figure 3.** Weight development of larval medaka exposed to the secondary effluents before and after chlorination for four weeks (n = 10). \*,  $p < 0.05$

increase of VTG induction in effluents after chlorination cannot be attributed only to the changes of estrogenic activity. In fact, the estrogenic activity of the chlorinated secondary effluent based on the *in vitro* test has been demonstrated to be lower than that of the effluent before chlorination (Wang 2004). As shown in Figures 2 and 3, the larval growth in chlorinated effluents was faster than that in secondary effluents after 3-week exposure ( $p < 0.05$ ), although it was significantly lower than that in the control after 4-week exposure ( $p < 0.05$ ). These results suggested that the water quality of the effluents after chlorination was improved, which was also demonstrated from the approximately 54.2% removal of PAHs (Table 2). Thus, VTG in chlorinated effluents was induced by the remaining estrogenic activity when the negative effects on the normal physiological metabolism became weak.

**Table 2.** PAH concentrations (ng/L) in secondary effluent before and after chlorination

	Control	Secondary effluents	Chlorinated effluents
Acenaphthene	ND	132	67
Phenanthrene	ND	291	156
Anthracene	ND	397	183
Fluoranthene	ND	61	ND
Dibenz(a,h)anthracene	ND	6	ND
Total		887	406

ND: no detection

In this paper, we reported that the phenomenon of VTG in fish was not induced by exposure to secondary effluents, while the induction of VTG of fish exposed to chlorinated effluents was improved. The mechanism underlying our observations needs to be further investigated.

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