Acute and early life stage toxicity of industrial effluent on Japanese medaka (*Oryzias latipes*)

Jinmiao Zha, Zijian Wang*

State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences,
P. O. Box 2871, Shuangqing Rd 18, Haidian District, Beijing, 100085, PR China

Received 5 April 2005; accepted 5 April 2005
Available online 21 June 2005

Abstract

To develop the whole effluent toxicity testing methods (WET), embryo larval stage toxicity test using Japanese medaka (*Oryzias latipes*) was conducted to evaluate an effluent from a banknote printing plant (BPP). The method is based on acute toxicity using endpoint of 96-h larval mortality and on chronic toxicity using endpoints such as the time to hatch, hatching success, deformity, growth rate, swim-up failure, accumulative mortality and sexual ratio. In test for 96-h larval mortality, LC50 (the concentration was lethal to 50% of newly hatching medaka larvae) was 72.9%. In chronic toxicity test, newly fertilized embryos (<5-h old) were exposed to 1%, 6.25%, 12.5%, 25%, 50% effluent concentrations and to 200 µg/l BPA in a 24-h static renewal system at 25 ± 1 °C until 15 day post-hatch. The results showed that all chronic endpoints were significantly different from the control at 50% dilution ($p < 0.01$). Embryos began to show lesions on 4th day at higher concentrations (12.5%, 25%, 50% BPP effluent concentrations). Treatment group of 25% dilution showed delayed time to hatch. A reduction in body weight was observed at 25% dilutions for males and females, respectively. Deformities were observed in newly hatched larvae at 25% and 50% BPP effluent concentrations. At 25% dilution, sex ratio of larvae was alternated and there was feminization phenomenon. We conclude that embryo larval stage test using medaka is feasible to evaluate both acute and chronic toxicities and potential endocrine disrupting activity of industrial effluents.

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Keywords: WET; Japanese medaka; Embryo; Larvae; Wastewater

1. Introduction

Industrial effluent discharges directly to surface waters or municipal sewage treatment plants. The

current standards for effluent discharges are not based on risk or impacts upon receiving waters. Many effluents are of a complex nature (Vyryan et al., 1999) and discharge a vast number and diversity of chemical substances to the receiving water, many of which are unknown (Trevizo and Nirmalak, 1999). In addition to chemical measurements, whole effluent toxicity (WET) methods generate the aggregate toxic

* Corresponding author. Tel.: +86 10 6284 9140; fax: +86 10 6292 3543.

E-mail address: wangzj@mail.rcees.ac.cn (Z. Wang).

effect of an effluent or its potential impact on receiving (ambient) waters. In recent WET tests, short-term chronic adverse effects were included and embryol larval survival and teratogenicity of fathead minnow (*Pimephales promelas*) were used as the test methods (USEPA, 2002). However, the test method is based on the total frequency of mortalities and gross morphological deformities. Many field studies suggest estrogenic effects on fish in polluted habitats, including reduced reproduction success (Petersen et al., 1997), reduced the levels of plasma estrogens in males (Johnson et al., 1988), and alternation in sex ratios (Lang et al., 1995). However, some reports showed anti-estrogenic activity on fish, for example masculinization of fish (Leblac et al., 1997), reduced gonad growth (Sanstrom et al., 1997), disrupted steroid synthesis by the ovary and altered peripheral metabolism of steroids (Van der Kraak et al., 1992). In addition, M74 syndrome (environmentally related disease 1974) in Baltic salmon and the equivalent Early Mortality Syndrome (EMS) in salmonids of North American Great Lakes were observed in fry and embryo (AFS, 1996). Some studies have reported that effluent from sewage treatment plants have both teratogenicity and estrogenic activity (Fuerchker et al., 2001; Montsrrat et al., 2001), while estrogenic activity has not been included in recent WET.

Medaka (*Oryzias latipes*) serves as an excellent fish model to determine acute and chronic toxicities, including endocrine disrupting activity of chemicals. Many reports have suggested that medaka is sensitive to toxicants and endocrine disrupting chemicals (Scholz et al., 2000; Metcalfe et al., 2001; Islinger et al., 2002), especially in early life stage.

The characteristics and effects of industrial effluents vary and the effluent guidelines need to be identified independently. For example, previous studies have demonstrated that effluent from sewage treatment plants (STP) were estrogenic to juvenile fish (McArdle et al., 2000; Julia et al., 2002). Kovacs et al. (1995) reported lower egg production and delayed reproduction in fathead minnow exposed to bleached kraft pulp mill effluents (BKME). However, no significant effects were observed in maturing rainbow trout exposed to effluent of a pulp and paper mill (Heuvel et al., 2002). USEPA is announcing a draft strategy that describes a process to identify industries for which effluent guidelines need to be revised or

developed (USEPA, 2002). There is little work being carried out on different types of industrial effluents in China.

In the present study, acute and chronic toxicity of effluent from a banknote printing plant (BPP) in medaka (*Oryzias latipes*) were assessed based on a variety of endpoints. The aims of the study are to determine the toxicity of effluents from BPP, the sensitivity of different endpoints, including lethal, sub-lethal, growth, deformities and sexual ratio, as well as the feasibility of using medaka as model animal in whole effluent toxicity testing methods.

2. Materials and methods

2.1. Sampling

Effluent samples were taken from a banknote printing plant in Beijing. The effluent was from the exit of a small-scale wastewater treatment facility using biological oxidation with active sludge. The wastewater is a combination of sewage wastewater and discharges from different processes in the industry.

The effluent sample was collected over a 24-h period according to composite sampling methods (USEPA, 2002). The sample was delivered to laboratory at 0–6 °C within a few hours. The effluent was filtered through a 60- μ m nylon plankton net to remove indigenous organisms. Then the effluent was kept frozen and thawed before use.

2.2. Fish culture

Japanese medaka (d-rR) was kindly provided from laboratory fish stock, Bioscience Center, Nagoya University, Japan. The brood stock were kept in dechlorinated tap water at a constant temperature of 25 ± 2 °C, with a photoperiod of 16:8 h (light:dark). The brood stock was fed newly hatched brine shrimp in the morning and flake food (Trea, Germany) in the afternoon. Eggs were collected daily from the females at less than 2 h after fertilization. Filaments attaching adjacent eggs were removed by using a sucker. Eggs were checked using a dissecting microscope to determine fertilization. Eggs with the migration of oil globules to the vegetal pole were deemed to be fertilized (Kirchen and West, 1976). Eggs were disinfected

by placing them in a 0.9% solution of hydrogen peroxide for 10 min (Marking et al., 1994), then placed in embryo rearing medium (ERM) (Kirchen and West, 1976) until initiation of exposure.

2.3. 96-h larval mortality experiment

Medaka larvae were exposed to 0% (control), 10%, 35%, 45%, 55%, 65%, 75%, 85%, and 100% of BPP effluent sample for 96 h to measure mortality. In each vial (300 ml), 10 newly hatched larvae and 100 ml test solution were added. Three replicates were performed for each treatment and control. All the vials were stored in an incubator at 25 °C. Larvae were examined daily for mortality and were fed with newly hatched brine shrimp only once. The experiment was carried out in a static renewal assay procedure (24 h renewal).

2.4. 15-day exposure experiments

Medaka embryos were exposed to BPP sewage effluent concentrations of 0%, 1%, 6.25%, 12.5%, 25% and 50%, respectively and to 200 µg/l bisphenol-A (BPA, Acros Organics) diluted from a stock solution of 20 mg/l BPA without organic solvent and stored in darkness at 4 °C. In each vial (300 ml), 10 fertilized eggs and 100 ml of test solution were added. There were 5 replicates per concentration gradient. The vials were stored in an incubator at 25 °C under a 16:8 h light:dark photoperiod. The test solution was renewed every second day until the embryos were hatched.

Newly hatched larvae and 500 ml test solution were placed into another vial (500 ml) at 25 °C under a 16:8h (light:dark) photoperiod. All larvae were fed with newly hatched brine shrimp three times each day. At larval phase, the test solution was renewed each day. The post-hatching exposure was continued for 15day post-hatch.

Both embryos and larvae cultured in embryos rare medium (ERM) were used as the control.

2.5. Endpoints

The embryological and larval stages of medaka development have already been well-documented (Iwamatsu, 1994). Embryo morphology was checked using a dissecting microscope and recorded with a

digital camera. Numbers of live, dead and lesions on all embryos were recorded daily. After hatching, numbers of live, dead and deformities of all larvae were monitored. Total body length (TBL) of newly hatched larvae was determined under a dissecting microscope with a micrometer eyepiece (Leica Wild MPS 52). At 15 days post-hatched, all larvae were sacrificed in buffered MS222 (500 mg/l) and total body length and wet weight were determined.

The genetic sex of medaka larvae were distinguished simply by observing its leucophore type and the color of the body under a dissecting microscope.

2.6. Data analysis

Treatment groups were compared with the control group in all cases. All data except sex ratio were assessed with a *t*-test using Microsoft Excel 2000 data analysis tools. The data on sex ratios were assessed by chi-square analysis. The critical level of statistical significance for all analyses was $\alpha=0.05$. LC50 values were calculated by probit transformation using the computer software SOFOTOX™ distributed by WINDOWCHEM™ Fairfield, CA, USA.

3. Results and discussions

3.1. Water quality parameters and chemical characteristics

The pH of BPP effluents was higher than control, but it did not exceed level of pH in culture water (see Table 1). DO of BPP effluents was significantly less than controls, however, it increased to levels of 5.8 to 6.4 mg/l to satisfy the requirement for fish test (USEPA, 2002). There were no significant differences of conductivity and total dissolved solid among treatments.

We investigate the residual levels of phthalic acid esters (PAEs) and organochlorine pesticide (OCPs) in BPP effluents. The result showed that concentrations of α -HCH, β -HCH, γ -HCH, δ -HCH and 4, 4'-DDT were higher in the treatments than in the control, but in ng/l level. Among PAEs, butyl benzyl phthalate (BBP) could be detected and its concentration in the effluent was 10.6 µg/l. No other chemical measurements have been done.

Table 1
Water quality parameters and chemical characteristics of BPP effluent and control

Sample	pH	TDS (mg/l)	CON (uS/l)	DO (mg/l)	α -HCH (μ g/l)	β -HCH (μ g/l)	γ -HCH (μ g/l)	δ -HCH (μ g/l)	4,4'-DDT (μ g/l)	BBP (μ g/l)
BPP	7.65	488	994	2.4	4.79	5.80	2.01	0.54	0.54	10.6
Control	6.97	545	1107	5.8	nd	nd	nd	nd	nd	nd

All data were mean data of two five replicates for each treatment.

nd showed less than determined limit.

DO, TDS, and CON showed respectively dissolved oxygen, total dissolved solids and conductivity.

HCH, DDT and BBP showed benzene hexachloride, dichloro-diphenyl-trichloroethane and Butyl benzyl phthalate, respectively.

3.2. Acute toxicity

For newly hatching medaka larvae exposed to effluent, the value of 96 h LC₅₀ was 72.9% effluent concentration. Below 50% effluent concentrations, larvae were not manifesting the acute toxicity. At concentration higher than 75%, no larvae survived.

Wang et al. (2002) has reported that the 96 h LC₅₀ indexes of carp and crucian carp exposed to effluents from STP are 69% and 79% respectively. Sun et al. (2001) reported that acute toxicity test using zebrafish was supplied to monitor the effluents from industrial discharge points. This experiment showed that effluents from several industries such as pesticide and petrochemical had significantly acute toxicity. However, we found that STP effluent from Beijing had no acute toxicity on medaka (data to be published).

3.3. Embryo toxicity

3.3.1. Embryo lesion

More than 90% of embryos were normal in effluent concentrations lower than 12.5%. Abnormalities of

embryos exposed to 12.5% concentration were observed at 4th day (Table 2). In previous works, some studies have reported that lesions of medaka embryos were observed at day 4 or day 5 after exposure to chemicals (Villalobos et al., 2000; Stephanie et al., 2001). Villalobos et al. (2000) found that day 4 or 5 after exposure was the time of formation of the liver. In our experiment, the results were agreed with previous results, indicating that there are toxicants in the effluent.

With the exposure time increasing, we found that percentage of lesions significantly increased in higher concentration groups. On day 9, the number of lesions of medaka embryos from treatment and control groups reached to maximum. Therefore for medaka, the best time for observation of lesions, when WET is applied, should be after 5 days. In 50% sewage effluent, the number of lesions increased to 48% at day 9 after exposure, which were significantly higher than in the control ($p < 0.01$) (Table 2). Percentage of lesions in 12.5% and 25% effluents were quite similar to that in 200 μ g/l of BPA (Table 2). Stephanie et al. (2001) found similar results (16% lesions) in medaka embry-

Table 2
Accumulative lesions rates at different day after exposure and their specific characteristics of embryos at 9 days

Concentrations (%)	Lesions rates (%)					Lesions characteristics
	Day 5	Day 6	Day 7	Day 8	Day 9	Day 9
0	2	4	8	8	8	PE(2) ^a , HR(1)
1	0	0	0	0	0	None
6.25	0	4	4	4	4	PE(1)
12.5	4	8	8	12	16*	PE(3), HR(2), HS(1)
25	6	8	12	16*	20*	PE(3), HR(3)
50	20*	24*	36**	40**	48**	PE(8), HR(5), HS(3)
200 μ g/l BAP	4	8	16*	16*	20*	PE(3), HR(2), HS(2)

^a The number of embryos exhibiting specific lesions is indicated in parentheses.

PE, HR, HS showed Pericardial edema, Hemorrhage, Hemostasis, respectively.

*, **, *** showed that significantly different from control were $p < 0.05$, $p < 0.01$, $p < 0.001$.

Fig. 1. Embryo lesions and larvae deformities of medaka (A) normal embryo, (B) lesion embryo (the arrow shows pericardial edema), (C) lesion embryo (the arrow shows hemorrhage), (D) normal larvae, (E) deformity larvae (the arrow shows curled of backbone on tail), (F) deformed larvae (the arrow shows blue sac).

os exposed to 200 $\mu\text{g/l}$ of BPA. The lesion rates from 12.5% and 25% BPP effluents, and that from 200 $\mu\text{g/l}$ of BPA were also significantly higher than that in the controls ($p < 0.05$) (Table 2).

Lesions characteristics could be different when exposed to effluent and to BPA (Fig. 1). For example, Stephanie et al. (2001) observed only hemorrhages when medaka were exposed to BPA and there was a lessening in the severity on day 9 when compared to

that on 4 days (Stephanie et al., 2001). In this study, lesions characteristics of medaka varied with different effluent concentrations (Table 2). Occurrences of pericardial edema and hemostasis were more prevalent above 12.5% effluent concentrations. Hirofumi et al. (2001) and Michelle and Metcalfe (1999) have also reported embryological lesions of medaka embryos when exposed to 4-NP and OP, respectively, but they did not describe the morphological details of the lesions. In addition, there have been reports since the early 90s of a wide spread occurrence of embryonic deformities in pelagic eggs of several fish species in coastal waters in the North Sea (Cameron et al., 1992). However, it is not yet clear whether these fluctuations reflect exposure to endocrine disrupters or other toxicants or may be caused by factors such as temperature (Cameron et al., 1992). These results showed that sewage effluent and chemicals could cause lesion of fish embryos. So, we thought that evidences of lesions characteristics may serve as an alternative indicator to identify chemical characteristics of effluents.

3.3.2. Indicators as time to hatch and hatching rate

In effluent concentrations lower than 12.5% and in BPA treatment, time to hatch was not different from that in control and embryos were hatched within 10 days. Time to hatch of embryos when exposed to 25% and 50% effluent concentrations increased significantly (Table 3) ($p < 0.05$ and $p < 0.01$ respectively). Only a few embryos were hatched after 10 days in 25% effluent concentration. However, there were embryos hatched even after 13 days in 50% effluent concentration. Prolonged time to hatch has been reported in zebrafish (*Danio rerio*) when exposed to sevin (carbaryl insecticide) (Nancy et al., 2002). Time to hatch

Table 3

Mean $\pm$ standard deviation ($n = 5$) of hatching success, time to hatch, deformity, total length at 0 day post-hatching, and swim-up failure in each concentrations

Concentrations (% effluent)	Hatching rate (%)	Time to hatch (d)	Deformity (%)	Body length (mm)	Swim-up failure (%)
0	96 $\pm$ 4.2	9.4 $\pm$ 0.3	4.2 $\pm$ 2.8	4.51 $\pm$ 0.17	4.9 $\pm$ 5.7
1	100	9.3 $\pm$ 0.1	0	4.53 $\pm$ 0.11	4.1 $\pm$ 4.8
6.25	100	9.2 $\pm$ 0.2	4.0 $\pm$ 5.0	4.44 $\pm$ 0.10	7.0 $\pm$ 6.1
12.5	82.6 $\pm$ 9.5*	9.4 $\pm$ 0.4	5.3 $\pm$ 3.4	4.31 $\pm$ 0.13	12.1 $\pm$ 5.2*
25	88 $\pm$ 5.7*	10 $\pm$ 0.5*	9.1 $\pm$ 5.6**	4.18 $\pm$ 0.21	19.6 $\pm$ 6.8**
50	72 $\pm$ 9.8***	11.1 $\pm$ 1.1**	44.4 $\pm$ 12.5***	3.98 $\pm$ 0.38	85.6 $\pm$ 24.4***
BPA	80.8 $\pm$ 5.0***	9.3 $\pm$ 0.3	5.0 $\pm$ 4.6	4.30 $\pm$ 0.19	16.8 $\pm$ 9.5**

*, **, *** showed that significantly different from control were $p < 0.05$, $p < 0.01$, $p < 0.001$.

could be an indicator for the specific toxicant at a critical exposure concentration.

Hatching rate in the control was $96 \pm 4.2\%$ (mean $\pm$ SD, $n=5$) (Table 3). For the embryos exposed to effluent concentrations of 1% and 6.25%, respectively, hatching rates were 100%. Hatching rates from 12.5 and 25% BPP were less than that in the control ($p<0.05$) (Table 3). Hatching rates from 50% BPP and 200 $\mu\text{g/l}$ of BPA were significantly less than the control ($p<0.001$) (Table 3). A reduction of hatching success, induced by an exposure to single chemicals, has been reported in a number of fish, such as in medaka when exposed to 4-Nonylphenol (NP) (Hirofumi et al., 2001) and fathead minnows when exposed to ethinylestradiol (EE2) (Reinhard et al., 2001). Reduction of hatching success was also observed in Baltic salmon (due to M74 contamination) and the equivalent EMS (Early Mortality Syndrome) syndrome in salmonids in North American Great lakes (AFS, 1996). In addition, Nagler and Cyr (1997) have reported exposed American plaice (*Hyppoglossoides platessoides*) males to marine sediments contaminated from St Lawrence Bay decreased hatching success of progeny in the semi-field study. So hatching rate could be a sensitive index for evaluation the toxicity of effluent on receiving waters.

3.4. Larvae toxicity

Deformities were observed in newly hatched larvae when exposed to higher effluent concentrations (Fig. 1). The backbone on the tail of some larvae curled and distorted and a few larvae had blue sac. Deformities rate at 25% and 50% effluent concentrations were

statistically different from control ($p<0.01$ and $p<0.001$, respectively). There were light significant differences in terms of deformity rates between 12.5% and the control ($p<0.05$) (Table 3). Therefore, effluent concentrations less than 12.5% and BPA should have no teratogenic effects in medaka. In our recent work (data to be published), medaka exposed to a STP effluent did not show blue sac, but it showed little backbone distortion. This result showed that the BPP effluent under concentrations greater than 12.5% contained teratogenic agents and that contained in STP may be different from those in BPP.

Swim-up failure is the consequence of chronic intoxication. Swim-up failure at 50% effluent concentration was $85.6 \pm 24.4\%$ and statistically different from control ($p<0.001$) (Table 3). Swim-up failure from 12.5% and 25% BPP effluents were significantly less than that in control ($p<0.05$, $p<0.01$, respectively) (Table 2). In addition, we found swim-up failure in treatment with 200 $\mu\text{g/l}$ of BPA was also significantly less than that in the control ($p<0.01$) (Table 3). Swim-up failure could be a supplementary indicator for chronic toxicity.

Body length of newly hatching larvae at effluent concentrations of 12.5%, 25% and 50% and that at 200 $\mu\text{g/l}$ BPA tended to be slightly lower than that in the control ($p<0.05$) (Table 3). Body length does not seem to be a sensitive indicator for early life stage exposure to BPP effluent.

Accumulative mortality rate of larvae increased to 100% when concentration of effluent was 50% at 15th day post-hatched (Table 4). Accumulative mortality of medaka at 15th day after exposure was also observed for higher concentration of a toxic chemical, such as

Table 4

Total length, body weight, accumulative mortality, and sex ratio as determined by gross examination of secondary sex characteristics at 15 day post-hatching in each concentrations

Concentrations (% effluent)	Length on day 15 (mm)	Body weight on day 15 (mg)	Accumulative mortality (%)	Sex ratio (σ : φ)
0	11.09 ± 1.67	11.85 ± 5.79	8.5 ± 4.6	23:20
1	11.109 ± 2.91	13.08 ± 6.54	6.3 ± 5.4	23:22
6.25	10.30 ± 2.21	$9.03 \pm 6.26^*$	12.6 ± 9.7	22:21
12.5	9.89 ± 2.71	$8.10 \pm 6.31^{**}$	$27.8 \pm 15.7^*$	15:17
25	9.33 ± 2.93	$7.81 \pm 5.21^{**}$	$38.5 \pm 24.9^{**}$	10:17 ^{a,b}
50	— ^a	— ^a	100 ^{***}	0:0
BPA	— ^a	— ^a	$68.7 \pm 30.4^{***}$	— ^a

—^a The number of larvae was not enough for statistical analysis.

*, **, *** showed that significantly different from control were $p<0.05$, $p<0.01$, $p<0.001$.

^b The statistic methods was chi-squared analysis.

4-NP (Hirofumi et al., 2001) or STP effluent in Beijing (data to be published). In the field studies, increasing mortality rates of juvenile chinook salmon (*Oncorhynchus tshawytscha*) occurred at sites where a STP outfall discharged to the Fraser River, British Columbia was reported (Birtwell et al., 1983). Smith and Suthers (1999) also reported that in a caging experiment of *T. taeniatus*, a large proportion (73%) died in one of the cages at the sewage outfall at Potter Point in 1996. These results showed sewage effluent caused increasing mortality rates of fish.

Body weights of larvae on day 15 post-hatch exposed to 12.5% and 25% BPP were slightly lower than that in the control ($p < 0.01$), while in 6.25% BPP effluent treatment groups, body weights of larvae on day 15 post-hatch were light less than that in the controls ($p < 0.05$) (Table 4). There was no difference in body length of larvae on day 15 among treatments. In contrast, in a semi-field study in Canada, American plaice from the St. Lawrence have shown a decrease in size (Nagler and Cyr, 1997). However, Jobling et al. (2004) have reported that immature carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) exposed to effluent from Chelmsford Sewage Treatment Works, Chelmsford, Essex, UK for 28 days showed no difference in the mean size (length or weight) of the fish between the treatment groups at the end of the trial. The results showed that growth, such as body length, could be served as, but not sensitive enough indicator for evaluation of the chronic toxicity for sewage effluents.

3.5. Endocrine disruption

Female and male medaka (d-rR) could easily be distinguished by using a dissecting microscope. The sexual ratio ($\sigma:\varphi$) of larvae at day 15 post-hatch was 23:20 in controls. Sexual ratio ($\sigma:\varphi$) of larvae exposed to 1% and 6.75% sewage effluent were 23:22 and 22:21, respectively. Ratios of female to male medaka ($\sigma:\varphi$) increased at 12.5% and 25% treatments. Sexual ratios were 15:17 and 10:17 at 12.5% and 25%, respectively (Table 4). Sex ratio in 25% BPP effluent was significantly different from that in the controls ($p < 0.05$) (Table 4). This result could be due to endocrine disruptors in the BPP effluents, or male fish could be sensitive to toxicants in BPP effluents. Lang et al. (1995) have shown change in

sex ratios of dab (*Limanda limanda*) from the North Sea with increased percentage of females and they believed that should be caused by contaminants. Some chemicals could certainly change sex ratio of fish. For example, Koger et al. (2000) have reported that ratio of female to male of medaka increased when embryos were exposed to 15 $\mu\text{g/l}$ of estradiol for 6 days.

4. Conclusions

It can be concluded that the chronic endpoints such as embryo lesions, hatching rate, time to hatch, deformities, swim-up failure could be supplanted in the recent WET protocol. When medaka is used as test model, the early stage of life should be easily used as sensitive tool for detection of endocrine disruptors in the effluents. In this case study, embryos began to show lesions on day 4 at 12.5%, 25%, 50% BPP effluent concentrations. At 25% dilution, it showed delayed time to hatch. At 25% dilution, the sex ratio of larvae was alternated. Discharge of BPP effluents with dilution would manifest potential ecological risk on receiving aquatic biology.

Acknowledgments

This work was supported by National Natural Science Foundation of China (20337020) and National Basic Research Program of China (2003CB415005).

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