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An attempt to detect contamination with estrogenic compounds in river water of urban area in Thailand and Malaysia using transgenic medaka

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Abstract—Contamination of estrogen-like substances (ELSs) in the river and coastal areas in Thailand and Malaysia was investigated using transgenic medaka, which indicates estrogenic contamination level as green fluorescence in its liver. Water samples were collected from two rivers in Thailand, four rivers in Malaysia, and three coastal areas (two in Thailand, one in Malaysia). Generally, no contamination was observed in headwater, river mouth, countryside, and coastal areas. On the other hand, detectable levels of contamination were observed in the river water of urban area. These suggest that contamination by estrogenic substances occurs in urban area, but these substances may be diluted and/or degraded to the undetectable levels until the water reaches river mouth. Relatively high levels of ELSs were observed at Sang Khep River mouth in Thailand and toxic effects were observed in urban area of Sang Khep River as well as urban area and the mouth of the Klang River in Malaysia.

Key words: endocrine disrupter, estrogen, transgenic, medaka, GFP

Introduction

Contamination of estrogen-like substances (ELSs, such as natural estrogen, synthetic estrogen, pharmaceutical products, and man-made chemicals) causes serious problems in aquatic environments. They interfere the endocrine system of living organisms in the ecosystem. Such contamination is mainly caused by human activities, in particular discharging of ELSs to river and coastal seas without treatment.

We have established a simple and rapid method using transgenic medaka to detect ELSs in aquatic environment (Kurauchi et al. 2005). Recently, we produced a transgenic medaka strain harboring green fluorescence protein (GFP) gene under regulation of the regulatory region of the gene of chorigenin H, a component of the egg envelope. This strain expresses green fluorescence in its liver by the stimulation of exogenous ELSs, and the intensity of the fluorescence shows a good correlation with ELS concentration of the rearing water., Thus, using the transgenic strain, ambient ELS level can be estimated by the intensity of the fluorescence. Compared with conventional methods, chemical analysis and *in vitro* bioassay, this transgenic medaka method has many advantages. For examples, this method can evaluate "real" bioactivities because the system utilizing the endogenous ELS-responsive elements and natural response to various ELSs can be monitored. We can also monitor bioconcentration, effects of metabolic conversion, additional and/or synergistic effects of compounds, etc., with low cost.

It is supposed that the main carrier of ELSs derived from human activities is river system, which delivers ELSs to costal seas. In this study, we investigated contamination of ELSs in river and coastal area in Thailand and Malaysia using the transgenic medaka, as the first step to evaluate the influence of contaminated fresh water flow on the water quality in coastal area. Our results indicated that generally ELSs levels are higher in urban area, where man-made wastes are discharged, than river mouth and coastal water.

Materials and Methods

Sampling sites

Sampling sites are shown in Fig. 1 and Table 1 summa-

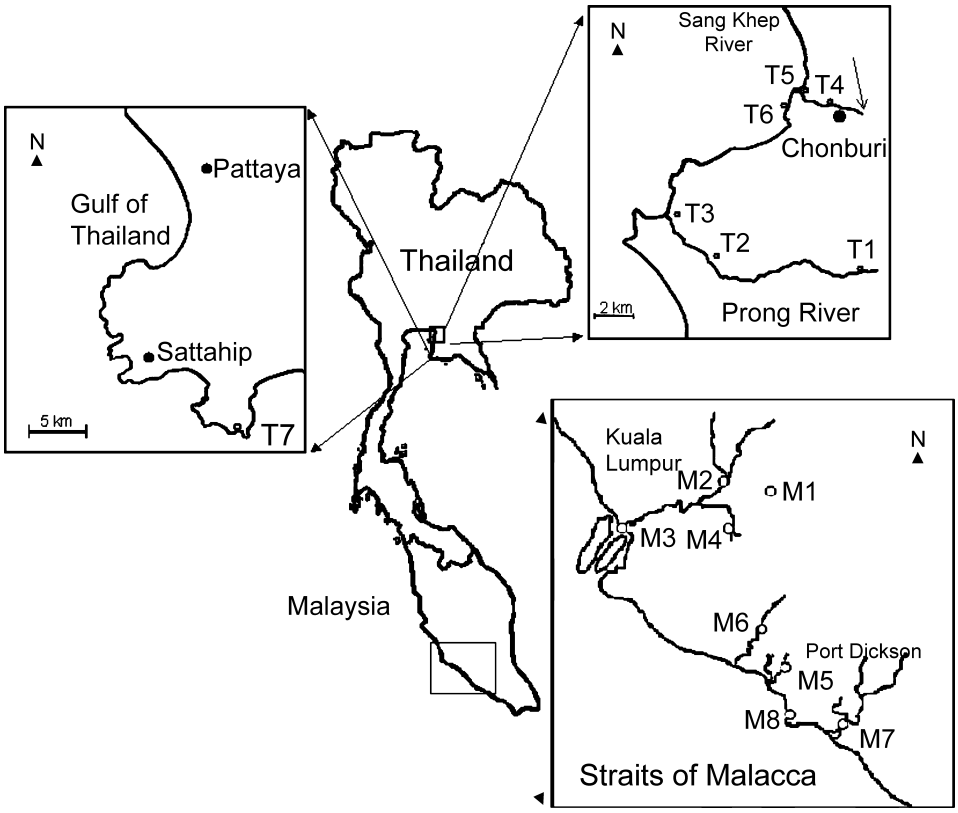

Fig. 1. Map of sampling sites in Malaysia and Thailand.
Detail information is summarized in Table 1.

Table 1. List of sampling sites, water conditions, and E2 equivalent.

Sampling site								Water condition				E2
Name	Name of river	Categoly	North Latitude	East Longitude	Date	Time	Weather	pH	temper-ature (°C)	Salinity (o/oo)	DO (mg/L)	equiva-lent (ppt)
Malaysia												
M1	Klang river	headwater	03.08.0821	101.50.171	2007.2.20	10:02	rainy	n.d.	25	n.d.	n.d.	U.D.
M2	Klang river	urban area	03.08.5378	101.41.433	2007.2.20	11:20	cloudy	n.d.	27	n.d.	n.d.	U.D.
M3	Klang river	river mouth	03.00.0843	101.23.232	2007.2.20	14:09	fine	n.d.	28	n.d.	n.d.	U.D.
M4	Klang river	urban area	03.01.020	101.42.159	2009.2.28	10:45	fine	4.6	28.7	0.1	n.d.	1–10
M5	Lukut river	countryside	02.35.190	101.49.588	2009.2.27	12:30	fine	5.5	27.4	3.5	n.d.	U.D.
M6	Sepang river	countryside	02.41.421	101.45.174	2009.2.27	12:50	cloudy	5.3	26.4	0.5	n.d.	U.D.
M7	Linggi river	river mouth	02.23.854	101.58.941	2009.2.27	10:30	fine	5.5	28.1	10.3	n.d.	U.D.
M8		coastal area	02.27.904	101.50.911	2009.2.27	11:20	fine	6.0	29.9	26.7	n.d.	U.D.
Thailand												
T1	Prong river	headwater	13.17.006	101.00.179	2008.7.3	n.d.	fine	n.d.	31	0	2.1	U.D.
T2	Prong river	urban area	13.17.255	100.56.404	2008.7.3	n.d.	fine	n.d.	30	0	3.0	1–10
T3	Prong river	river mouth	13.18.845	100.55.062	2008.7.3	n.d.	fine	n.d.	33.3	9.2	7.0	U.D.
T4	Sang Khep river	urban area	13.22.481	100.59.267	2008.7.4	12:50	n.d.	n.d.	n.d.	n.d.	n.d.	4–50
	Sang Khep river	urban area			2008.10.9	12:55	fine	7.6	30.3	0	2.5	U.D.
T5	Sang Khep river	river mouth	13.22.446	100.58.802	2008.10.9	12:40	fine	7.16	34	3.2	1.0	60–500
T6		coastal area	13.22.254	100.58.570	2008.10.9	13:10	fine	7.66	32.8	20	2.5	U.D.
T7		Fish farm	12.36.158	100.57.229	2008.10.11	10:45	fine	8.0	30.5	30.2	2.2	U.D.

n.d.: not detected, U.D.: under detection level.

rizes the detail of each sampling site.

Extraction and concentration of estrogenic substances from water sample

One or three liters of each water sample were extracted and concentrated as described previously by Kurauchi et al. 2005. Briefly, water samples were filtrated with glass fiber filter and estrogenic substances were extracted using Sep-pak C-18 cartridge (Waters, MA). Absorbed estrogenic substances were eluted with 7 mL of methanol followed by 7 mL of diethyl ether. After evaporation of methanol and diethyl ether, extracts were dissolved in dimethyl sulfoxide (DMSO) to give 800–1,000 times concentrates against original volume.

Fish: ChgH-GFP transgenic medaka

In this study, ChgH-GFP transgenic medaka line was used. This transgenic medaka harbors green fluorescent protein (GFP) gene controlled by regulatory region of medaka choriogenin H gene. The useful features of this medaka are follows: Green fluorescence in liver is induced by the stimulation of estrogenic substances in culture water and the intensity of the fluorescence depends on the concentration of the estrogenic substances. (for more detail, see Kurauchi et al. 2005)

Exposure conditions and estimation of E2 equivalent in environmental water

Exposure solution was prepared by diluting each concentrated sample with embryo culture medium (ECM: 0.1% NaCl, 0.003% KCl, 0.004% $CaCl_2/2H_2O$, 0.016% $MgSO_4/7H_2O$) to give 0.5, 2.5, 5, and 10 times concentration from the original water. Each exposure solution contained less than 2% of DMSO as a final concentration. 17β-estradiol (E2) solution was used as a positive control.

Fig. 2 summarizes the procedure of exposure test and detection system of fluorescence. Five yolk-sac larvae (one-day post hatch: 1-dph) were exposed to 9 mL of exposure solution in plastic Petri dish (6 cm in diameter) for 24 h at 26°C.

After exposure, intensity of green fluorescence in liver was recorded using a stereomicroscope (MZFL III, Leica Microscopy Systems, Heerbrugg,Switzerland) equipped with a color digital cooled charged-coupled device (CCD) camera (VB-7010, KEYENCE, Osaka, Japan). As described previously (Kurauchi et al. 2005), the CCD camera system calculates exposure time to automatically give a constant light intensity and the reciprocal of exposure time represents the concentration of estrogenic substances in culture water as an E2 equivalent.

E2 equivalent of water samples was estimated by comparing various concentrates of environmental sample with various concentrations of standard E2 as follows: As shown in Fig. 3 and Table 2, the reciprocals of individuals exposed to 10 ppt E2 were almost the same as those of medium control (1% and 2% DMSO) and never exceeded 10, representing significant difference from those exposed to 50, 100, and 250 ppt E2. Therefore, we decided that the value 10 of reciprocal was the criterion to judge whether the water contained biologically affective estrogenic contamination. For example, in case of M4 sampling site, reciprocal of individual 1 which exposed to ×10 concentrate was 30 (more than 10), meaning that the ×10 concentrate contained more than 10 ppt E2 equivalent (Fig. 3 and Table 2). And reciprocal of the individual 1 exposed to ×5 concentrate was 1 (less than 10), meaning ×5 concentrate contained less than 50 ppt E2 equivalent. As a result, environmental water at M4 contained more than 1 ppt, and less than 10 ppt E2 equivalent, (1 ppt<, <10 ppt). In case that some fish marked over 10 of reciprocal but some did not for the same sample water, we adopted larger reciprocal to estimate E2 equivalent.

In case that the reciprocal was less than 10 using ×10 concentrate of certain sample water, we judged as under the detection limit.

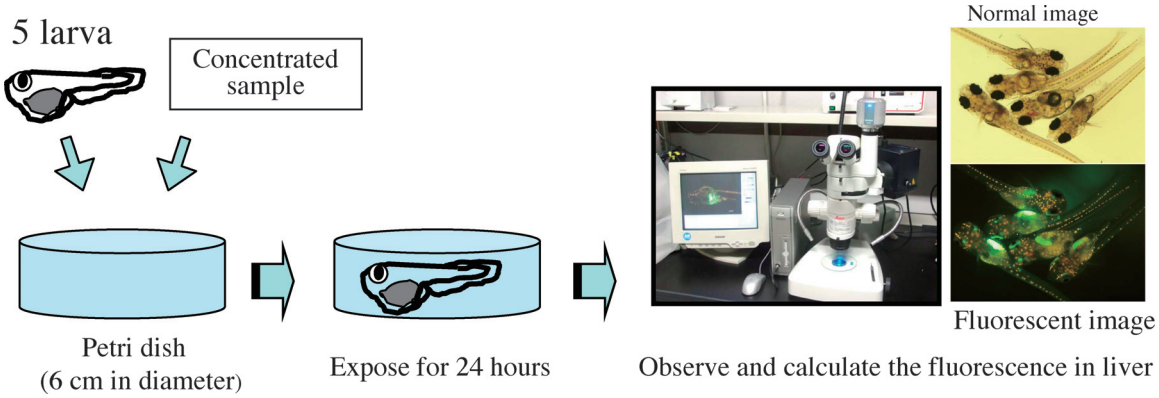

Fig. 2. Scheme for detection of estrogen-like substances.
Five transgenic medaka larva were exposed to 9 mL of concentrated water sample for 24 h with Petri dish (6 cm in diameter). Then, the intensity of green fluorescence in liver was measured with fluorescence microscope and equipped computer system.

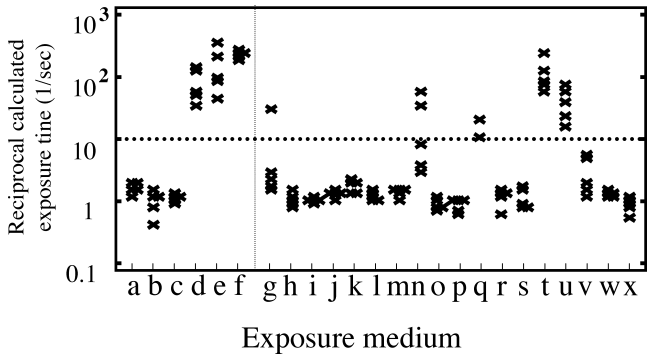

Fig. 3. Reciprocal of environmental water sample.
Reciprocal was calculated with exposure time obtained by fluorescence microscope and computer system to give a constant light intensity. Each symbol, x, represents each individual. a: 1% DMSO, b:2%DMSO, c: 10 ppt 17β-estradiol (E2), d: 50 ppt E2, e: 100 ppt E2, f: 250 ppt E2, g: M4 (×10 concentrate), h: M4 (×5), i: M5 (×10), j: M6 (×10), k: M7 (×10), l: M8 (×10), m: T1 (×10), n: T2 (×10), o: T2 (×5), p: T3 (×10), q: T4: (×2.5, 080704), r: T4 (×1, 080704), s: T4 (×2.5, 081009), t: T5 (×0.25), u: T5 (×0.17), v: T5 (×0.1), w: T6 (×10), x: T7 (×10).

Table 2. The list of reciprocal of each individal.

Culture medium or sampling site	Concentration ratio	Reciprocal of each fish				
		fish 1	fish 2	fish 3	fish 4	fish 5
1% DMSO		1.5	2	1.5	2	1.1
2% DMSO		1.5	1.1	1.1	0.43	0.77
E2 10 ppt		1	1.1	1.2	1.3	1.2
E2 50 ppt		130	50	130	60	35
E2 100 ppt		90	400	45	230	100
E2 250 ppt		230	250	280	200	250
M4	×10	30	3	1.8	2.3	1.5
	×5	1	1.5	0.91	1.2	0.83
M5	×10	1	1	1	0.9	1.2
M6	×10	1	1.5	1.3	1.3	1.3
M7	×10	2	2	2.3	1.3	1.3
M8	×10	1	1	1.2	1.3	1.5
T1	×10	1.5	1.5	1.5	1.5	1.2
T2	×10	60	35	9	3.5	2.8
	×5	0.83	1.2	0.83	0.77	1.1
T3	×10	1	1	1	0.77	0.67
T4	×5	dead	dead	dead	dead	dead
(080704)	×2.5	11	20	dead	dead	dead
	×1	1.3	1.2	1.3	1.5	0.67
T4	×10	dead	dead	dead	dead	dead
(081009)	×2.5	0.91	0.83	0.83	1.8	1.5
T5	×0.25	250	120	80	90	60
	×0.17	60	23	80	40	15
	×0.1	1.5	5	6	2	1.3
T6	×10	1.1	1.1	1.2	1.2	1.3
T7	×10	0.56	0.91	1.1	1	0.83

dead: Reciprocal was not obtained bedcause fish was dead during exposure period.

Results

Response of transgenic medaka, ChgH-GFP medaka, to standard chemical

In our previous paper, it was reported that the transgenic medaka, ChgH-GFP medaka, responded to not only 17b-estradiol (E2), but also natural estrogen, such as estron, and manmade estrogenic chemicals, such as ethinyl estadiol and nonylphenol and that the estrogenic activity of these substances could be represented as E2 equivalent value (Kurauchi et al. 2005). In this study, total estrogenic activity of environmental sample was represented as E2 equivalent

value. Therefore, first of all, we confirmed the responsibility of this transgenic medaka by exposing to the standard chemical, 17β-estradiol (E2), for 24 h. As shown in Figure 3, the reciprocal exposed to 10 ppt E2 was less than 2 and has no difference with that exposed control medium, DMSO. On the contrary, the reciprocals of 50, 100, and 250 ppt E2 (from 35 to 400) were one or two order of magnitude higher than that of 10 ppt E2. These results suggest that there was a threshold concentration of estrogenic substances for inducing GFP, in other ward, for generating biological effects. In this study, the concentration was between 10 and 50 ppt E2, and we judged estrogenic contamination occurred when the reciprocal exceeded 10.

Experiment 1: Klang river in Malaysia (Sampling sites M1–M3)

Environmental water sample was collected from mountain area (M1 in Fig. 1), urban area (M2, center of Kuala Lumpur), and river mouth (M3, Port Klang). The water from M1 was clear and had no odor. After exposure with ten times concentrate (×10) of M1 water samples, all medaka individuals were alive and did not express any green fluorescence in their liver, indicating that the water contains less estrogenic compounds than the detection limit (data not shown). On the other hand, exposure to the waters from M2 and M3, which were muddy and had malodor, was lethal even at ×1 concentration (same concentration as original water). Therefore, we could not evaluate estrogenic contamination (data not show). Thus, in M2 and M3 toxicity was more serious problem than estrogenic contamination.

Experiment 2: M4–M8 in Malaysia

The water of M4, an urban area of Klang river, did not induce green fluorescence in four of five fish tested. However, one individual showed distinct fluorescent with ×10 concentrate and no fluorescent was observed with ×5 concentrate, indicating possible existence of slight amount of estrogenic substances (more than 1 ppt, less than 10 ppt). (Fig. 4 and Table 2) No contamination was observed at countryside of Lukut river (M5) and Sepang river (M6), and river mouth of Linggi river (M7). At the coastal area (M8: Marine Station of University Putra Malaysia, UPM), no fluorescence over the threshold was detected.

Experiment 3: T1–T7 in Thailand

The offshore water sample (T7) was clear and no estrogenic contamination was detected (Table 2). In contrast, we detected estrogenic activities in samples from two river (Prong river and Sang Khep river) in Chon Buri province (Table 2).

Prong River: Headwater (T1) was clear and estrogenic activity was undetectable. The water sample in urban area (T2: Bansean city) induced distinct fluorescence in two of five tested fish, indicating slight contamination of estrogenic substances (more than 1 ppt, less than 10 ppt). (Fig. 4 and Table 2) At river mouth (T3), no contamination was detected.

Sang Khep River: Water sampling was performed at urban area (T4: Chon Buri city). In both cases, all fish subjected to exposure test were dead during exposure period using ×5 or ×10 concentrate, indicating some toxic compounds were contaminated in this site. With ×2.5 concentration collected on the sampling date, July 4 2008, three of five fish were dead and remaining two fish showed green fluorescence. With ×1 concentration, green fluorescence was not observed. Thus, slight contamination (less than 50 ppt E2 equivalent) was observed on July 4 2008. On the other hand, ×2.5 concentrate collected on October 9 2008 did not show any toxic effects and did not induce green fluorescence. Different from other environmental water sample, the water sample from Sang Khep river mouth (T5) induced intensive green fluorescence. (Fig. 4) Namely, even 6 times diluted sample (×0.17 concentrate) induced intense green fluores-

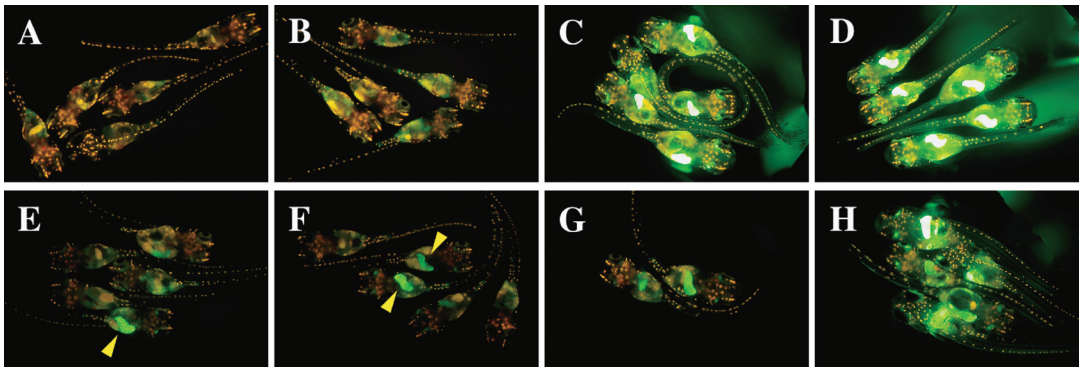

Fig. 4. Fluorescent images of transgenic medaka.
FIve ChgH-GFP transgenic medaka larva were exposed for 24 h.
A: exposed to 1% of DMSO, B: 10 ppt of 17β-estradiol (E2), C:50 ppt of E2, D: 100 ppt of E2, E: 10 times concentrate (×10) of M4, F: ×10 of T2, G: ×2.5 of T4 (4 July 2008), H: ×0.17 of T5. Green fluorescence was not induced by 10 ppt of E2 (B). On the contrary, intense green fluorescence was induced by 50 and 100 ppt of E2 (C and D).The fish indicated by arrowhead in E and F are whose reciprocal exceeded 10 (See Table 2). The reciprocal of all fish in G and H exceeded 10. (See Table 2)

cence, suggesting serious contamination with estrogenic substances (more than 60 ppt E2 equivalent) in this area. To investigate the influence of this serious contamination on coastal environment, coastal water sample (T6) near the river mouth was investigated. Ten times more concentrated water from T6 did not induced green fluorescence, meaning that estrogenic level was under the detection limit (Table 2).

Discussion

Pawlowski et al. (2004) reported that the level of estrogenic substances in the River Rhine in southern Germany was approximately 12 ppt of E2 equivalent with YES assay. Shapell (2006) reported that estrogenic activity in wetland, ponds, and river in U.S.A. was 0.3 ppt of E2 equivalent or lower with E-screen assay. In Japanese river, the contamination level of estrogenic substances is also similar, for example 0–7.2 ppt of E2 detected with GC/MS (MOE 2002) and 2.63–7.26 ppt of E2 equivalent detected with ELISA (Suzuki et al. 2003). Matsuoka et al. (2005) reported that the level of E2 equivalent in Muko river, located urban area in Japan, was usually less than 10 ppt, and that occasionally it elevated to 32.9 ppt using E-screen assay and Ishikawa cell-ALP assay. In this study, the river water of M4 and T2 contained less than 10 ppt of E2 equivalent and that of T4 contained less than 50 ppt of E2 equivalent, indicating that the estrogenic contamination level in urban river water is almost same among countries. Even in such low level of contamination, chronic exposure may affect on sex differentiation as reported by Seki et al. (2005). They reported that when medaka embryo was exposed from 12 h post fertilization to adult, abnormal sex differentiation and induction of vitellogenin was observed at 8.66 ppt of E2 at the lowest.

The river water of T5, however, was seriously contaminated and the level of E2 equivalent was more than 60 ppt equivalent. In such highly contaminated aquatic environment, gonad formation may be affected during short exposure period. Indeed, Hirai et al. (2006) reported that by exposing to 33.5 or 140.6 ppt of E2, testis-ova were observed at 14-day post hatch (dph) or 12-dph medaka larva, respectively. They also mentioned that some medaka were sex-transformed by the exposure of 140.6 ppt of E2 in early life stage.

Estrogen-like activity was not detected in coastal area examined in this study. It is likely that no special source of ELS near the sampling points of the coastal area, and ELS from the urban area, some of which exhibited high ELS levels, may have been diluted and/or degraded. At least for natural estrogens, degradation by microorganisms in the river water has been reported (Matsuoka et al. 2005, Jurgens et al. 2002).

For the effect of estrogenic contamination on wild life, the level of estrogenic substances and duration of exposure are critical factors. In actual aquatic environment, both factors are always changing daily, weekly, and monthly (Suzuki et al. 2003, Matsuoka et al. 2005). In addition, environmental conditions can change due to various causes such as the change in the source of contamination, urbanization, change in human population, the amount of river flow, weather, temperature, and so on. Therefore, continuous monitoring of estrogenic contamination and observation of wildlife are required. For such purpose, the transgenic fish system is useful because we can monitor the status of contamination, including the effect of chronic exposure and bioconcentration, by only keeping the fish in the test water, although closed culture system is necessary to use transgenic fish.

As reported by Inoue and Takei (2002), medaka (*Oryzias latipes*) has adaptability to seawater. Thus, in this study, we used transgenic yolk-sac larvae of this species and successfully estimated estrogenic contamination both fresh water sample and seawater sample. As mentioned above, continuous monitoring is required to investigate the effects on wildlife. For continuous monitoring, use of adult fish, which are more tolerant to various environmental factors including salinity, current etc., is preferable. It is also expected that the result obtained using adults are more stable than those by developing larvae. Now we are trying to establish continuous monitoring system with adult transgenic medaka.

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