Development of on-site fish exposure system placed in water quality monitoring stations along a river

T. Higashitani*, K. Miyajima*, N. Nakada*, M. Yasojima*, H. Tanaka and Y. Suzuki***

*Water Quality Research Team, Public Works Research Institute, 1-6, Minami-hara, Tsukuba, Ibaraki, 305-8516 Japan (E-mail: *higasi44@pwri.go.jp*)

**Research Center for Environmental Quality Control, Kyoto University, 1-2, Yumihama, Ohtsu, Shiga, 520-0811 Japan

Abstract Estrogen-like substances have been suspected to cause feminization of wild fish in rivers in Japan. To elucidate the influence of estrogen-like substances on fish in river, we have started to develop the on-site continuous fish exposure system using medaka *Oryzias latipes* that were placed in water quality monitoring stations along a river. Adult male medaka were exposed to the river water in a glass exposure tank placed in the monitoring stations. Flow rate of water and water temperature were controlled at 30 L/hour and 26 °C respectively, and a light: dark cycle was maintained 16:8 hours. A commercial diet free from phyto-estrogens was fed 4 times in a day using automatic feeder. After 2-week exposure, hepatic vitellogenin concentration of each male medaka was measured. The exposure tests were repeatedly performed at both the upstream and the downstream of sewage treatment plants along the River Tama which is a representative urbanized river in Japan. At the control site Haijimabashi monitoring station, vitellogenin was not detected in male medaka. On the other hand, at the Ishihara monitoring station which is the most downstream in this test area, every male medaka were produced vitellogenin in the test performed in the spring of 2004. As the results of the water quality analysis, it could be inferred that the estrone derived from effluents of sewage treatment plants caused the feminization of male medaka. The reason why the concentrations of the estrone and the estrogenic activity using DNA recombinant yeast varied in proportion to the electric conductivity of river water measured at the water quality monitoring station. Furthermore, after continuous 2-week exposure, the vitellogenin production of male medaka was reduced similar to the decrease of the concentrations of the estrone and the estrogenic activity of river water.

Keywords Estrogenic activity; feminization; medaka; on-site fish exposure system

Water Science & Technology **Vol 52 No 12 pp 275–282** © IWA Publishing 2005

Introduction

To investigate whether influence of estrogen-like substances for fish is actually occurring in Japanese rivers, the Ministry of Land, Infrastructure and Transport, Japan (MLIT) had conducted river field survey targeting on carp *Cyprinus carpio* during 1998 to 2001 (MLIT, 2002). The results had confirmed the production of vitellogenin that was reported to be a biomarker of feminization syndrome of male fish, occurring in 26% male carp irrespective of the season. This fact suggests that feminization of wild fish may occur in Japanese rivers usually, though the cause has not yet been elucidated. However, the relationship between the ratio of the male carp whose serum vitellogenin was detected and the average estrogenic activity of the river water by DNA recombinant yeast has a weak positive correlation (Tanaka *et al.*, 2003; Higashitani *et al.*, 2003a).

On the other hand, to investigate whether male carp are really feminized by estrogen-like substances in effluent from sewage treatment plants in Japan, exposure tests were performed using carp in water tanks that were receiving effluent from a sewage treatment plant. As the results, the vitellogenin concentration in male carp exposed to the effluent increased with time in the early spring of 2000, while significant level of vitellogenin in male carp could not be observed in the other tests performed in the same tank, though

the estrogenic activities were almost the same level throughout tests (Higashitani *et al.*, 2003b).

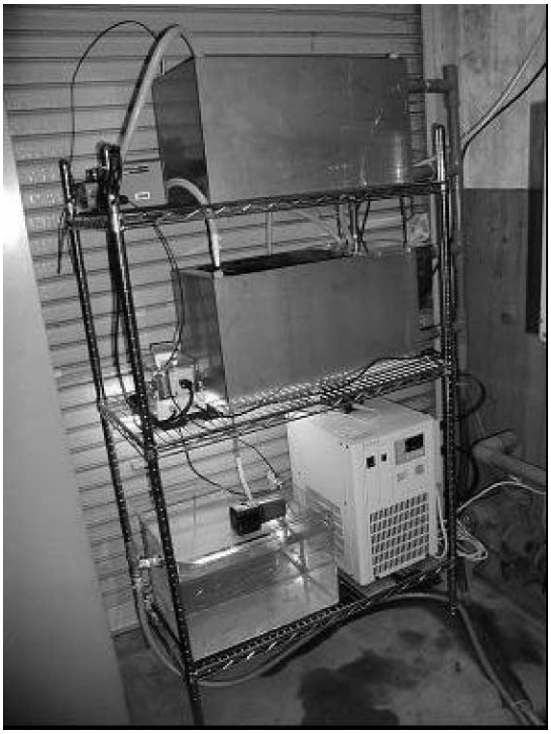
On the usual river field survey similar to the survey by MLIT, it is difficult to clear the amount of estrogen-like substances that taken in the fish body through a lifetime. The bioaccumulation of estrogens in periphytons and benthic invertebrates was observed from 100 to 1000 times higher than river water (Takahashi *et al.*, 2003). Therefore, it is very difficult to elucidate the influences that have been occurring in the water environment. Furthermore, on the carp exposure tests mentioned above, as the test conditions of water temperature and light: dark cycle were changed corresponding to the season, carp might be showing a seasonal physiological reaction in respect of the production of vitellogenin.

The purpose of this study is therefore to develop the exposure system on the field survey that could regulate a test condition similarly to the indoor laboratory experiment. For this end, medaka exposure tests were performed using new on-site fish exposure system.

Materials and methods

On-site fish exposure system

To detect the influence of estrogen-like substances on fish in river water, the fish exposure system is required to keep the test conditions constant except for the water quality. Furthermore, as for river water to use, it is desirable to be fresh. Therefore, it was decided that the on-site fish exposure system (Picture 1) be installed in the water quality monitoring station administered by MLIT. At the water quality monitoring station, river water was pumped up, and water temperature, pH, dissolved oxygen, electric conductivity and turbidity in the water were measured automatically every hour. To monitor the water quality automatically, profuse river water is pumped up to the reservoir of the water quality monitoring apparatus in the interval of 30 minutes, and the surplus water was drained



Picture 1 On-site fish exposure system

directly from this reservoir. Therefore, it is decided this surplus water is applied to the fish exposure system.

When the reservoir water quality monitoring apparatus is filled with river water, the float sensor installed in the reservoir responds, and the exclusive pump is operating to supply fresh river water to the fish exposure system. While the reservoir is empty, the float sensor and pump do not operate.

The river water, which is supplied by pump, is sent to the initial tank of the exposure system. The aim of the initial tank is to eliminate trash and grit, and to regulate the volume of flowing water by adjusting the capillary, which it is made to drop to the second tank. The second tank is connected to a constant temperature circulator, and water temperature is maintained while water circulated. After that, the river water dropped into the fish exposure glass tank through the siphon installed in the second tank. At the fish exposure glass tank, the drainage hole is covered with stainless steel grid mesh, so that medaka wasn't washed away with the water. Because inflow from the second tank runs down the force by the siphon well, this mechanism is expected to wash away the remaining feed and deposits. And, the drainage of the surplus water of the first tank, the second tank and the exposure glass tank was eliminated together in the drainpipe. The lighting sequence of the illumination is controlled by a program time switch, and a commercial diet free from phyto-estrogens was fed four times in a day using an automatic feeder. Furthermore, the thermostat heater is used at the third fish exposure glass tank to ensure that the water temperature of the exposure tank is kept the constant.

The necessary condition on the on-site fish exposure system is that it is possible that a test condition is regulated in the same way as the test aquariums of the indoor experiments. Flow rate of water has been controlled at 30 L/hour using the first tank, and water temperature controlled at 25 °C using constant temperature circulator. The light:dark cycle and diet are also set in the same way of the indoor test. Furthermore, test period set for 2 week followed the indoor tests (Ministry of the Environment, Japan, 2003).

Exposure design

The exposure tests were repeatedly performed both upstream and downstream of sewage treatment plants along the River Tama, which is a representative urbanized river in Japan. Haijimabashi monitoring station has not received effluents of sewage treatment plant, but both Hinobashi and Ishihara monitoring station received the effluents respectively of three and eight sewage treatment plants (Figure 1).

Medaka were selected for the following reasons: because the breeding is easy, medaka were utilized on various exposure experiments (MOE, 2003). Therefore, much basic information about the medaka is known well. On the first and second test, adult male medaka of about seven months are provided from Metocean Environment Inc. which researched the influence of chemical compounds to medaka with MOE. On the third test, young adult male medaka d-rR strain of about three months breeding in our laboratory is

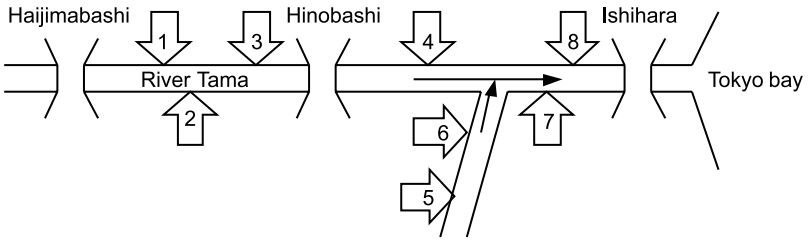


Figure 1 Schema of the test area along the River Tama. A number in arrow shows sewage treatment plants counted from the upstream side of the river

exposed. Medaka exposure tests were performed in winter and summer of 2003, and spring of 2004 (Table 1).

Vitellogenin and water quality analysis

Hepatic vitellogenin of each male medaka was measured by the medaka vitellogenin ELISA-kit (Transgenic Inc., Japan). When the exposure test finished, medaka was brought back to the laboratory, and measured body length, body wet weight, and liver weight before the day is over. Immediately every liver was ground in the micro tubes contained the sample buffer of the ELISA-kit. Liver extract samples after centrifugal separation were kept in the deep freezer till the vitellogenin analysis. Hepatic vitellogenin was measured due to the protocol of the ELISA-kit.

Estrogenic activity was measured regularly by DNA recombinant yeast assay (Yakou *et al.*, 1999; Miyamoto *et al.*, 2002). Estrone, 17β-estradiol and 17α-ethinylestradiol were measured by LC/MS/MS (Japan Sewage Works Association, 2001).

Results and discussion

Vitellogenin synthesis of male medaka

At the Ishihara monitoring station which is the most downstream in this test area, every male medaka at both 2-week and 4-week exposure produced hepatic vitellogenin in the test performed in the spring of 2004 (Figure 2). As the result of the water quality analysis, estrone and estrogenic activity were 6.7 ~ 33.1 ng/L and 5.3 ~ 27.9 ng/L (E2-equivalent), respectively (Table 2). The concentrations of the estrone and the estrogenic activity varied in proportion to the electric conductivity of river water measured at the water quality monitoring station (Figure 3). Furthermore, at 4-week exposure, the vitellogenin production of male medaka was reduced similar to the decrease of the concentrations of the estrone and the estrogenic activity of river water. To compare the course of the concentration of estrogenic activity and electric conductivity, it could be thought the estrogen which derived from the effluents caused the feminization of male medaka (Figure 3).

On the second test, male medaka did not produce vitellogenin at the same Ishihara monitoring station. During the test periods, because the test area was visited by a typhoon, the amount of river water temporarily increased (Figure 4). As the result, the concentration of both estrogens and electric conductivity were reduced. On the first test, male medaka did not produce vitellogenin at Hinobashi monitoring station, too (Figure 4). On this test, electric conductivity was lower than that of the Ishihara monitoring station on the third test, therefore the concentration of estrogens seemed to be lower.

No vitellogenin production of male medaka was detected at Haijimabashi monitoring station throughout the tests on the River Tama. Haijimabashi monitoring station does not

Table 1 Exposure design and fish

	Test series	Duration of exposure	Medaka				
			Number	Age (months)	Body length (mm)	Body wet weight (mg)	Liver wet weight (mg)
Test 1	Haijimabashi	2 weeks	20	7	29.0 ± 1.7	479.8 ± 89.6	13.9 ± 4.1
	Hinobashi	2 weeks	20	7	28.0 ± 1.0	356.5 ± 40.4	5.7 ± 1.4
Test 2	Ishihara	2 weeks	20	7	29.7 ± 1.5	468.6 ± 61.1	9.1 ± 1.9
Test 3	Haijimabashi	2 weeks	8	3	22.0 ± 1.6	220.5 ± 41.9	6.6 ± 2.3
		4 weeks	7	3	24.6 ± 0.7	230.6 ± 24.5	3.7 ± 0.8
	Ishihara	2 weeks	9	3	22.3 ± 1.5	219.9 ± 35.2	7.0 ± 1.9
		4 weeks	10	3	24.7 ± 0.9	234.5 ± 17.3	4.3 ± 1.3

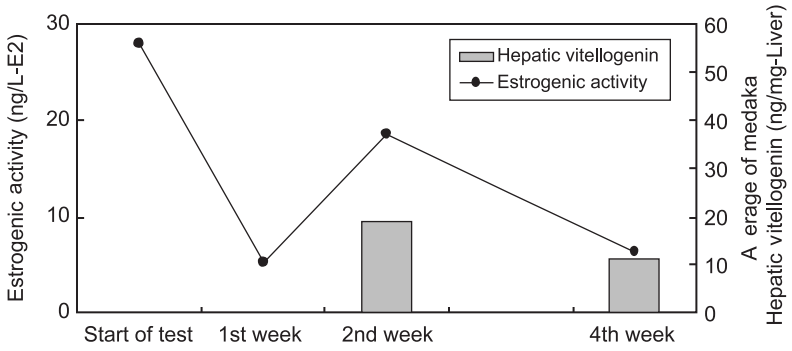


Figure 2 Hepatic vitellogenin productions in male medaka and changes of estrogenic activity at the Ishihara monitoring station on the test 3 in the spring of 2004

receive the effluent of sewage treatment plant, electric conductivity was changed almost about 13 mS/m.

As mentioned above, it could imply the factor which makes male medaka produce the hepatic vitellogenin, the ratio of effluents of sewage treatment plants to occupy in the river water, and then the concentration of estrogens to be high.

The Ministry of Environment, Japan had announced the influence of the nonylphenol and 4-t-octylphenol that exerts it on the medaka officially (MOE, 2003).

In that report, the concentration of hepatic vitellogenin from 10 to 20 ng/mg-liver level observed at the Ishihara monitoring station is equal to the influence of 20 ng/L of nonylphenol. Furthermore, when medaka were exposed during 60-day post hatch to 20 ng/L nonylphenol, conversion from male to female seemed to be appearing.

According to previously published reports, estrone, 17β-estradiol, synthetic estrogen 17α-ethinylestradiol and the degradation products of alkylphenol ethoxylate surfactants (e.g. nonylphenol) are major causes that have estrogenic effects in effluents of sewage treatment plants (Desbrow *et al.*, 1998; Harries *et al.*, 1997). On these researches, to clear

Table 2 Changes of estrogenic activity (EA), 17β-estradiol (E2), estrone (E1) and 17α-ethynylestradiol (EE2) in the river water through the tests

			Start of test	1st week	2nd week	4th week
Test 1	Haijimabashi	EA	0.16	0.16	0.12	–
		E2	ND	ND	ND	–
		E1	ND	ND	ND	–
		EE2	ND	ND	ND	–
	Hinobashi	EA	12.54	6.59	10.62	–
		E2	1.5	Tr (1.0)	1.7	–
		E1	21.9	12.2	15.2	–
		EE2	ND	ND	ND	–
Test 2	Ishihara	EA	6.31	8.29	6.56	–
		E2	ND	Tr (0.4)	ND	–
		E1	5.8	8.1	4.4	–
		EE2	ND	ND	ND	–
Test 3	Haijimabashi	EA	0.35	0.24	0.24	Tr (0.05)
		E2	ND	ND	ND	ND
		E1	ND	ND	ND	ND
		EE2	ND	ND	ND	ND
	Ishihara	EA	27.94	5.34	18.65	6.37
		E2	2.7	Tr (0.8)	4.6	ND
		E1	33.1	6.7	24.3	7.0
		EE2	ND	ND	ND	ND

ND: under the minimum limit of detection
Tr: above the minimum limit of detection, under the minimum limit of quantifying

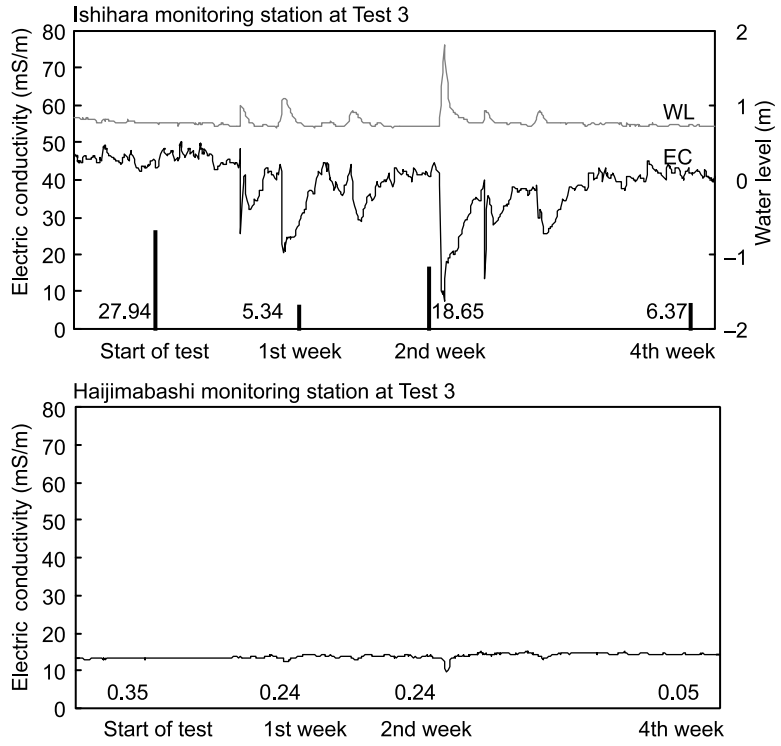


Figure 3 Changes of electric conductivity at both the Ishihara monitoring station (top) and Haijima monitoring station (bottom) at Test 3. Bar and figure show the estrogenic activity

the factor of influence for fish, a fractionation system, combined with an in vitro assay for detecting estrogenic activity, was developed in order to isolate and identify the major estrogenic chemicals in the effluents. To develop the on-site fish exposure system, it might be necessary for methods to isolate and identify the estrogen-like substances in the water.

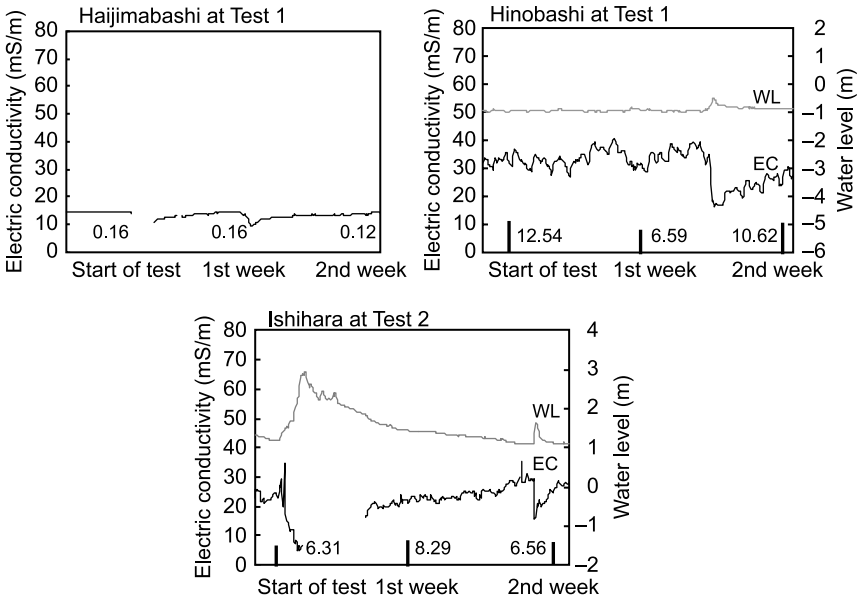


Figure 4 Changes of electric conductivity at Test 1 and 2. Bar and figure show the estrogenic activity

Improvement of on-site fish exposure system

In spite of estrone and estrogenic activity being higher than those of Ishihara monitoring station at the second test, vitellogenin of male medaka had not produced at Hinobashi monitoring station at the first test. Because the exposure test at Hinobashi monitoring station was performed in winter, water temperature was controlled at from 20 to 22 °C. It is known that the sensibility of the medaka to the chemicals was influenced by the difference of water temperature (Iwamatsu, 1997). Therefore, if the test condition regulated well, vitellogenin production might be detect at Hinobashi monitoring station at the first test because electric conductivity at Hinobashi monitoring station was not so low compared with Ishihara monitoring station. It could imply that the influence to the medaka and the causes could be explained by maintained water temperature accurately.

Conclusions

To elucidate the influence of estrogen-like substances on fish in river, we have started to develop an on-site continuous fish exposure system using medaka (*Oryzias latipes*) that were placed in water quality monitoring stations along a river. But, to clear the influence to fish in the water environment, the on-site fish exposure system needs more accurately control of the test condition.

At the Ishihara monitoring station which is the most downstream in this test area, every male medaka at both 2-week and 4-week exposure produced hepatic vitellogenin in the test performed in the spring of 2004. To compare the course of the concentration of estrogenic activity and electric conductivity suggested that the estrogens which derived from the effluents caused the feminization of male medaka in the river.

To develop the on-site fish exposure system, it might be necessary for methods to isolate and identify the estrogen-like substances in the water.

Acknowledgements

The authors thank Prof. Koji Arizono, Faculty of Environment in Prefectural University of Kumamoto and Prof. Akihiko Hara, Graduate School of Fisheries Sciences in Hokkaido University for their valuable suggestions and advice regarding this study. We also thank to the staff of Keihin Office of River, Kanto Regional Development Bureau, Ministry of Land, Infrastructure and Transport for providing the test area, and the staff of Metocean Environment Inc. for providing the test fish. We also thank to the staff in Public Works Research Institute for their great assistance in this study.

References

Desbrow, C., Routledge, E.J., Brighty, G.C., Sumpter, J.P. and Waldock, M. (1998). Identification of estrogenic chemicals in STW effluent, 1. Chemical fractionation and in vitro biological screening. *Environmental Science and Technology*, **32**(11), 1549–1558.

Harries, J.E., Shehan, D.A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J.P., Tylir, T. and Zaman, N. (1997). Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environmental Toxicology and Chemistry*, **16**(3), 534–542.

Higashitani, T., Tamamoto, H., Miyamoto, N., Yasojima, M. and Tanaka, H. (2003a). Feminization of wild carp and carp exposure tests using sewage treatment plant effluents. In *Preprints of the 4th IWA Specialized Conference on Assessment and Control of Hazardous Substances in Water – ECOHAZARD 2003*, Aachen, 14-17 September.

Higashitani, T., Tamamoto, H., Takahashi, A. and Tanaka, H. (2003b). Study of estrogenic effects on carp (Cyprinus carpio) exposed to sewage treatment plant effluents. *Wat. Sci. Tech.*, **47**(9), 93–100.

Iwamatsu, T. (1997). *The integrated book for the biology of the medaka*, Daigaku-kyoiku-shuppan, Japan (in Japanese).

Japan Sewage Works Association (2001). *Manual on water quality analysis for endocrine disrupting chemicals in sewerage system*, 2nd edn, Japan Sewage Works Association, Tokyo, Japan (in Japanese).

Ministry of Land, Infrastructure and Transport (2002). Results of the field survey targeting on EDCs in the water environment (in Japanese). http://www.mlit.go.jp/river/press/200207_12/021212/siryo1.pdf.

Ministry of the Environment, Japan (2003). Medaka *Oryzias latipes*: Development of test methods and suitability of medaka as test organism for detection of endocrine disrupting chemicals. http://www.env.go.jp/chemi/end/medaka.html.

Miyamoto, N., Tanaka, H., Tamamoto, H. and Komori, K. (2002). Fractionation method for estimating the cause of estrogen-like activity in sewage. *CDROM of proceedings of WEFTEC'2002*.

Takahashi, A., Higashitani, T., Yakou, Y., Saitou, M., Tamamoto, H. and Tanaka, H. (2003). Evaluating bioaccumulation of suspected endocrine disrupters into periphytons and benthos in the Tama river. *Wat. Sci. Tech.*, **47**(9), 73–76.

Tanaka, H., Higashitani, T., Miyamoto, N., Tamamoto, H., Komori, K., Tsujihara, K. and Ohnishi, Y. (2003). Estrogenic activity of river water and feminization of wild carp in Japan. In *CD-ROM of WEFTEC'03 Conference Proceedings, 76th Annual Technical Exhibition and Conference*.

Yakou, Y., Takahashi, A., Higashitani, T. and Tanaka, H. (1999). Measurement of estrogen-like activity in wastewater using recombinant yeast. *Environmental Engineering Research*, **36**, 199–208 (in Japanese).