Part2 Hazard assessment of tributyltin (TBT) and 4-nonylphenol (NP)

The Fish Working Group in Japan has developed vitellogenin (VTG) assay, and partial life-cycle test (PLC) for screening purposes, and full life-cycle test (FLC) for definitive test with medaka (*Oryzias latipes*), and also conducted hazard assessment of chemicals listed in SPEED '98 using these test methods. The activities of the group were reported at the special session of the Endocrine Disrupter Testing and Assessment (EDTA) in 2002, and the data are shown at the end of this part. In this part, tributyltin (TBT) and 4-nonylphenol (NP) (*see Appendix II-II*) that is a known environmental alkyl phenol are featured.

1. 4-Nonylphenol (NP)

1-1. *In vitro* assays

1-1-1. Competitive binding assay to medaka estrogen receptor á (ERá)

Competitive receptor binding assay was performed using medaka (*Oryzias latipes*) and human estrogen receptor á ligand binding domains expressed in E. coli., and the binding affinities of nonylphenol (mixture), 4-*t*-octylphenol, 4-*t*-pentylphenol and 4-*t*-butylphenol to these recombinant receptors were measured. Release of the radiolabelled ligand from medaka ERá depending on the concentrations of 17â-estradiol, nonylphenol, and 4-t-octylphenol were observed (Fig.1). Their relative binding affinities (RBA) to both medaka and human receptors compared with 17â-estradiol was summarized in Table 1. It was found that alkyl phenols with branched alkyl chain bound to medaka ERá according to their chain length and RBA values were about several hundreds times stronger than those to human ERá. Especially, nonylphenol (mixture) and 4-*t*-octylphenol had high receptor binding abilities and their RBA values were about 1/10 and 1/15 of 17â-estradiol, respectively. Other branched alkylphenols tested also showed relatively high receptor binding abilities. The RBA values of 4-*t*-pentylphenol and 4-*t*-butylphenol were 1.1 and 0.15, respectively, and they were hundreds times greater than those to human ERá as in the case with nonylphenol and 4-*t*-octylphenol. On the other hand, linear alkylphenols bound to medaka ERá weakly. Their RBA values were less than 0.1% when compared with 17â-estradiol and almost alike to those to human ERá.

Furthermore, the binding abilities of nonylphenol were examined for medaka ERâ, and ERá from other fish, carp (*Cyprinus carpio*) and mummichog (*Fundulus heteroclitus*) with a same procedure. The RBA values of nonylphenol to medaka ER â and mummichog ERá were 1/110 and 1/200 of 17â-estradiol, respectively.

However, it bound to carp ERá weaker than to other fish ERs (RBA ~ 0.1%). In conclusion, alkylphenols with branched bulky alkyl chains showed relatively high binding affinities to estrogen receptors from fish compared with those to human estrogen receptor.

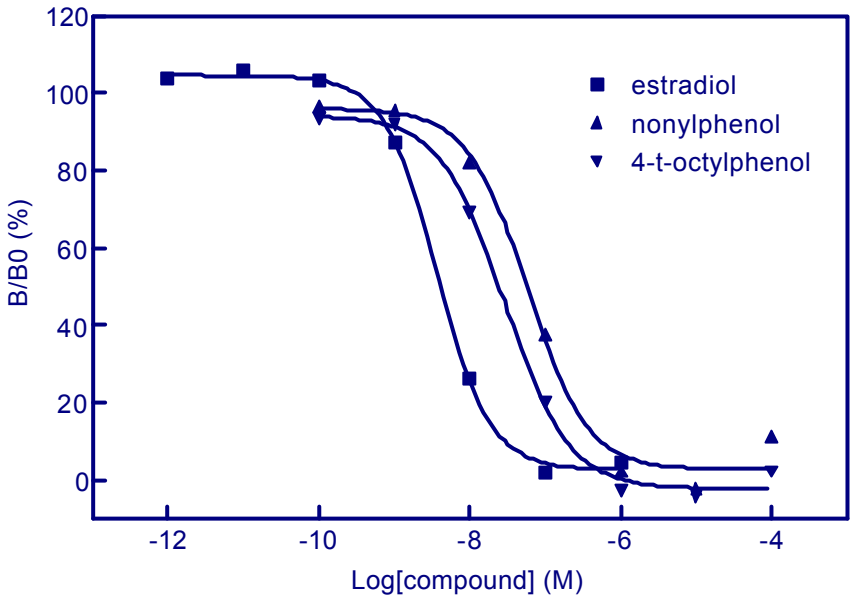

Fig. 1 Dose-response curves of 17â-estradiol and alkylphenols in the radioligand receptor binding assay using [^{3}H]17â-estradiol and medaka ERá expressed in *E. coli*.

Table 1 IC_{50} values and relative binding affinities (%) of alkylphenols to medaka and human estrogen receptors α ligand binding domain

Chemical substances	Medaka *1		Human *2	
	IC_{50} values (M)	Relative binding affinity(%)	IC_{50} values (M)	Relative binding affinity (%)
Estradiol	4.8×10^{-9}	100	2.1×10^{-9}	100
Nonylphenol(mixture)	7.9×10^{-8}	8.1	3.4×10^{-6}	0.061
4-*t*-Octylphenol	3.2×10^{-8}	16	6.6×10^{-6}	0.032
4-*t*-Pentylphenol	3.9×10^{-7}	1.1	4.1×10^{-5}	0.0051
4-*t*-Butylphenol	3.0×10^{-6}	0.15	1.6×10^{-4}	0.0013
4-*n*-Nonylphenol	1.1×10^{-6}	0.038	4.2×10^{-6}	0.050
4-*n*-Octylphenol	5.3×10^{-6}	0.077	1.1×10^{-5}	0.020
4-*n*-Pentylphenol	5.5×10^{-6}	0.084	—	—
4-*n*-Butylphenol	6.5×10^{-6}	0.066	8.8×10^{-5}	0.0024

*1: Measured four times for nonylphenol (mixture) and 4-*t*-octylphenol, and three times for other chemical substances.

*2: Measured three times for all chemical substances.

1-1-2. Reporter gene assay

Transcriptional activities of alkylphenols mediated by medaka ERá were measured using HeLa cells transiently co-transfected with both receptor expression and reporter (firefly luciferase) vectors. It was found that transactivation function presented by EC50 value of nonylphenol was three hundred times weaker than 17â-estradiol (Fig. 2).

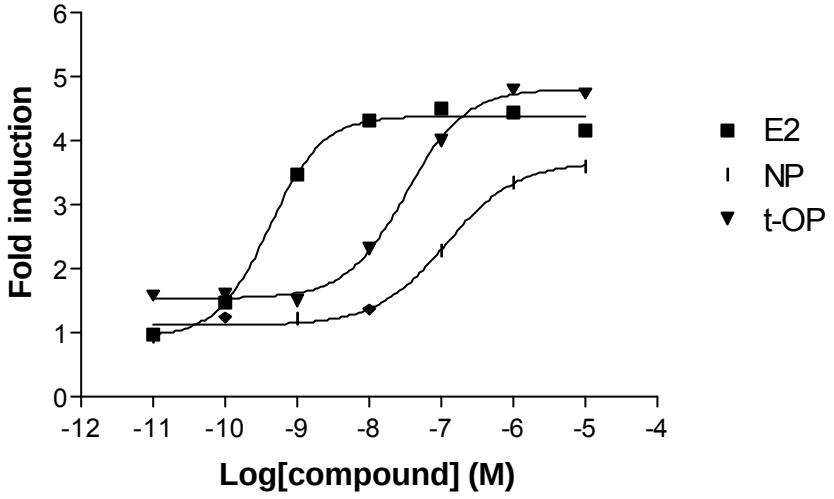

Fig. 2 Reporter gene transactivation assay using HeLa cells co-transfected with medaka estrogen receptor expression and reporter vectors.

1-2. *In vivo* studies using medaka

1-2-1. Screening

Medaka vitellogenin assay

The estrogenic potency of nonylphenol (mixture; NP) and 4-*t*-octylphenol (4-*t*-OP) was evaluated using in vivo vitellogenin (precursor of egg yolk protein) synthesis in medaka. About 3-month-old medaka (respectively 8 females and males / treatment) were exposed to 5 test concentrations of each substance (NP; 7.40, 12.8, 22.5, 56.2 and 118ìg/L, 4-t-OP; 12.7, 27.8, 64.1, 129 and 296ìg/L as mean measured concentrations) under flow-through conditions for 21 days. 17â-estradiol (E2; 100ng/L) was tested as positive control. Daily observation was made to examine mortality and abnormal behavior and appearance during the exposure period. At the end of exposure, the livers of exposed fish were removed, and vitellogenin concentration in each liver was measured.

In either NP or 4-*t*-OP study, any death or particular symptom was not observed through the exposure period. The hepatic vitellogenin concentrations in males were increased in a concentration-dependent manner, and a statistically significant induction was observed at ≥ 22.5 μg/L for NP study and ≥ 64.1 μg/L for 4-t-OP study (Fig.3).

These results suggested that both NP and 4-*t*-OP could cause vitellogenin synthesis in the livers of male medaka through their estrogenic activities.

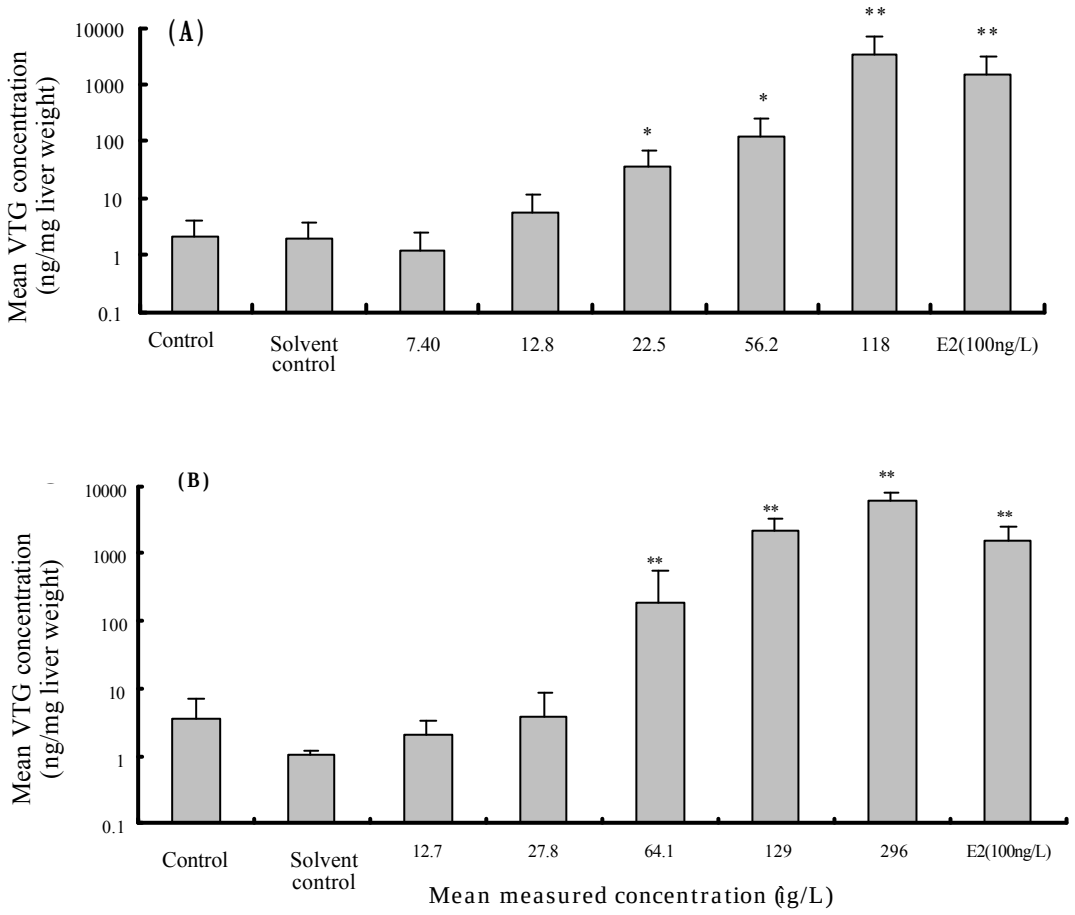

Fig. 3 Vitellogenin (VTG) concentrations in the livers of male medaka (*Oryzias latipes*) in NP study (A) and 4-*t*-OP study (B).Data is shown as mean±standard deviation. * and ** denote significant differences at $p < 0.05$ and $p < 0.01$, respectively.

Medaka partial life test

This test was performed to assess endocrine disrupting effects of nonylphenol

(mixture; NP) and 4-*t*-octylphenol (4-*t*-OP) on sex differentiation of medaka. Medaka (60 eggs/treatment) were exposed to 5 test concentrations of each substance (NP; 3.30, 6.08, 11.6, 23.5 and 44.7 ìg/L, 4-*t*-OP; 6.94, 11.4, 23.7, 48.1 and 94.0 ìg/L as mean measured concentrations) under flow-through conditions from fertilized eggs to 60-day posthatch. During the exposure period, hatching, posthatch mortality, and abnormal behavior and appearance were observed daily. At the end of exposure (at 60-day posthatch), the total length and body weight of all the surviving fish were measured, and the sex of each individual was determined from the appearance of secondary sex characteristics. Furthermore, 20 individuals from each treatment group were randomly sampled, and then their livers and gonads were removed for vitellogenin measurement and gonadal histology.

In either NP or 4-*t*-OP test, any particular effect on hatching of fertilized eggs and posthatch mortality was not observed at the concentrations tested. As for growth of fish at 60-day posthatch in the NP test, however, a significant decrease was observed in both total length and body weight in the 44.7 ìg/L treatment, and in body weight in the 23.5 ìg/L treatment. This result suggests that NP adversely affects the growth of medaka. In the 4-*t*-OP test, no growth reduction was observed at the concentrations tested. The sex ratio estimated from the appearance of secondary sex characteristics of the surviving fish at 60-day posthatch was significantly skewed toward female at ≥ 23.5 ìg/L in NP test and ≥ 48.1 ìg/L in 4-*t*-OP test (Table 4 and 5). Furthermore, gonadal histology showed that the fish in ≥ 11.6 ìg/L NP treatment groups and ≥ 11.4 ìg/L 4-*t*-OP treatment groups had testis-ova as shown by the presence of oocytes in the testis (hereinafter referred to as testis-ova, or, Table 2 and 3). The hepatic vitellogenin concentrations in males exposed to ≥ 11.6 ìg/L NP and ≥ 11.4 ìg/L 4-*t*-OP were significantly increased(Fig.4).

These results indicate that both NP and 4-*t*-OP exert estrogenic effects on sex differentiation of male medaka, and suggest that the Lowest-Observed-Effect Concentrations (LOECs) of NP and 4-*t*-OP for feminization of the appearance of their secondary sex characteristics were 23.5 ìg/L and 48.1 ìg/L, respectively, and that the LOECs of them for induction of testis-ova and vitellogenin were 11.6vg/L and 11.4 ìg/L.

Table 2 Sex ratios as determined by gross examination of secondary sex characteristics of medaka (*Oryzias latipes*) at 60-day posthatch in NP test, and by their gonadal histology.

NP concentration (ìg/L)	Secondary sex characteristics N: Number of fish Sex ratio (male:female)		Gonadal histology N: Number of fish Testis	Ovary	Testis-ova	
Control	55	25 : 30	20	8	12	0
Solvent control	57	27 : 30	20	10	10	0
3.30	59	27 : 32	20	9	11	0
6.08	59	25 : 34	20	10	10	0
11.6	57	28 : 29	20	9	7	4*
23.5	58	11 : 47**	20	2	9	9**
44.7	60	1 : 59**	20	1	15	4**

* and ** denote significant differences at $p < 0.05$ and $p < 0.01$, respectively.

Table 3 Sex ratios as determined by gross examination of secondary sex characteristics of medaka (*Oryzias latipes*) at 60-day posthatch in 4-*t*-OP test, and by their gonadal histology.

4-*t*-OP concentration (ìg/L)	Secondary sex characteristics N: Number of fish Sex ratio(male:female)		Gonadal histology N: Number of fish Testis	Ovary	Testis-ova	
Control	55	25 : 30	20	10	10	0
Solvent control	56	21 : 35	20	9	11	0
6.94	55	26 : 29	20	10	10	0
11.4	56	25 : 31	20	8	11	1
23.7	48	13 : 35	20	8	10	2
48.1	56	13 : 43**	20	7	10	3*
94.0	54	0 : 54**	20	1	15	5*

* and ** denote significant differences at $p < 0.05$ and $p < 0.01$, respectively

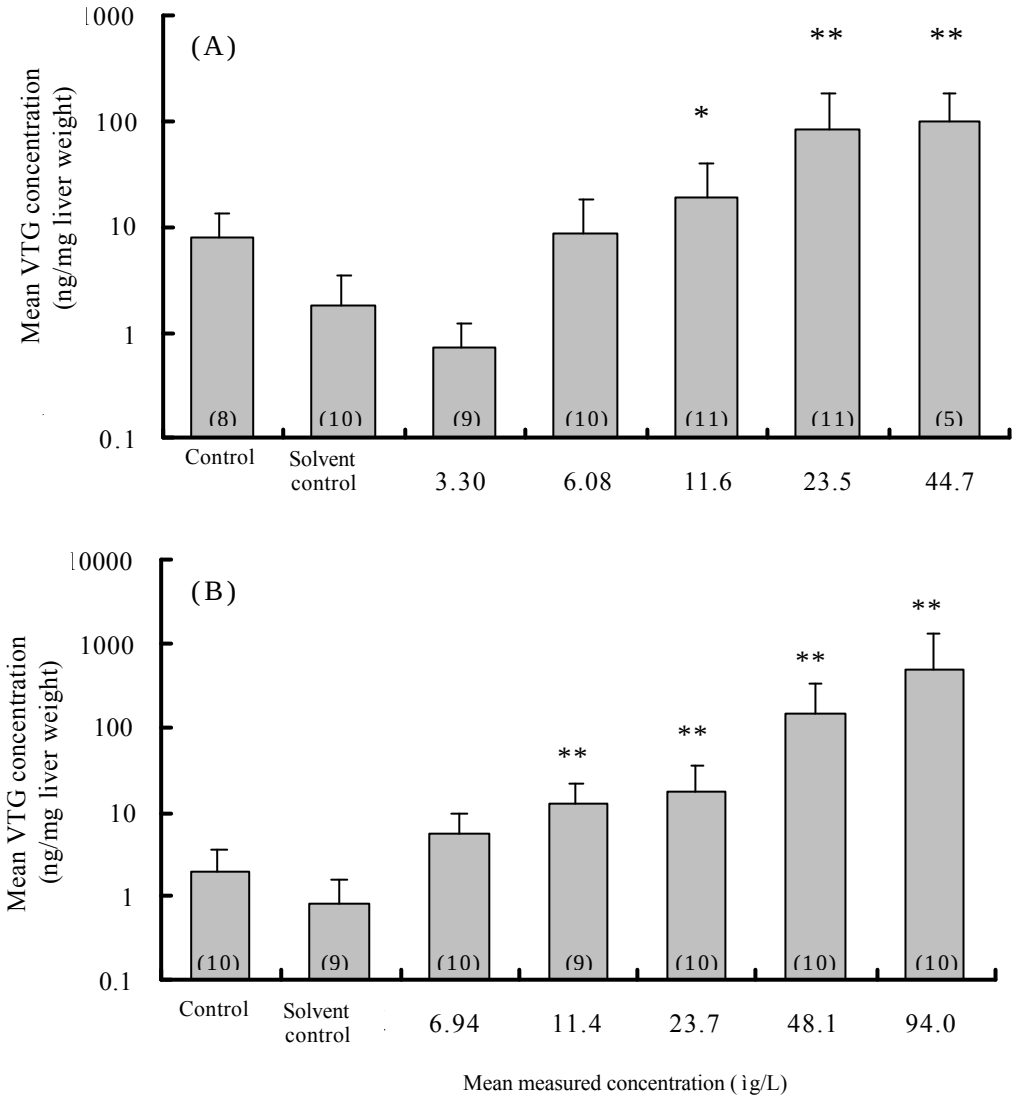

Fig. 4 Vitellogenin (VTG) concentrations in the livers of male medaka (*Oryzias latipes*) at 60-day posthatch in NP test (A) and 4-*t*-OP test (B). Data is shown as mean±standard deviation. Numbers in parentheses indicate the number of fish. * and ** denote significant differences at $p < 0.05$ and $p < 0.01$, respectively.

1-2-2. Definitive test (medaka full life-cycle test)

This test was conducted to elucidate chronic toxicity and endocrine disrupting effects of nonylphenol (mixture; NP) on the life cycle of medaka. Medaka (60 eggs/treatment) were exposed to mean measured NP concentrations of 4.2, 8.2, 17.7, 51.5 and 183ìg/L under flow-through conditions from fertilized eggs to 104-day

posthatch. During the exposure period, hatching, posthatch mortality and abnormal behavior and appearance were observed daily. At 60-day posthatch, phenotype sex was determined from the appearance of secondary sex characteristics, and histological observation of gonad was made for 20 fish per treatment. Furthermore, at 70-day posthatch, 6 mating pairs in the 2 low treatment (4.2 and 8.2 µg/L) and the controls and solvent controls were selected. No pairs from the 51.5 µg/L treatment and only 3 pairs from the 17.7 µg/L treatment could be selected due to a skewed sex ratio and/or the limited number of surviving fish. The eggs spawned from each female were counted daily and assessed for viability until 104-day posthatch. The fertilized eggs spawned on 102- and 103- day posthatch of the parental generation were also exposed in the same system until 60-day posthatch, and effects were examined.

The 183 µg/L treatment significantly reduced the embryo survival and swim-up success of the F_0 fish. The cumulative mortality of the F_0 fish from swim-up to 60-day posthatch were significantly increased in the 17.7 and 51.5 µg/L treatments. No concentration-related effect was observed on the growth of fish at 60-day posthatch. However, the sex ratio estimated from the appearance of their secondary sex characteristics was completely skewed toward female in the 51.5 µg/L treatment (Table 4).

Additionally, gonadal histology showed that the fish in 17.7 and 51.5 µg/L treatments had testis-ova (Table 4). The sex ratio of the F0 fish in the 51.5 µg/L treatment was completely skewed toward female, subsequently the mating pairs from ≤ 17.7 µg/L treatments were selected at 70-day posthatch, and their fecundity and fertility were observed daily until 103-day posthatch. Fecundity was unaffected by any of the treatments examined. The mean fertility in the 17.7 µg/L treatment was reduced to 76% of that in the controls, although no statistically significant differences were determined (Fig.5). Overall, these results suggest that the lowest-observed-effect-concentration and no-observed-effect-concentration of NP through the life cycle of the F_0 medaka were 17.7 µg/L and 8.2 µg/L, respectively. In the progeny generation (F_1), no significant effects were observed on hatching, posthatch mortality, or growth at the concentrations tested (4.2 to 17.7 µg/L). However, induction of testis-ova in the gonads of the F_1 fish at 60-day posthatch was observed in both the 8.2 µg/L and 17.7 µg/L (Table 5). This result suggests that NP could have significant effects on reproductive potential of the F_1 medaka at lower concentrations than 17.7 µg/L.

Table 4 Sex ratios as determined by gross examination of secondary sex characteristics of the F_0 medaka (*Oryzias latipes*) at 60-day posthatch and by their gonadal histology.

NP concentration (ìg/L)	*N* : Number of fish	Sex ratio (male:female)	Gonadal histology *N* : Number of fish		
			Testis	Ovary	Testis-ova
Control	20	9 : 11	9	11	0
Solvent control	20	8 : 12	8	12	0
4.2	20	12 : 8	12	8	0
8.2	20	13 : 7	14	6	0
17.7	20	9 : 11	5	11	4
51.5 a	20	0 : 20	0	12	8

a: The sex ratio obtained from gonadal histology differed significantly from that of the solvent control at $p < 0.001$.

Table 5 Sex ratios as determined by gross examination of secondary sex characteristics of the F_1 medaka (*Oryzias latipes*) at 60-day posthatch and by their gonadal histology.

NP concentration (ìg/L)	*N* : Number of fish	Sex ratio (male:female)	Gonadal histology *N* : Number of fish			
			Testis	Ovary	Testis-ova	
Control	59	28 : 31	20	7	13	0
Solvent control	54	26 : 28	20	11	9	0
4.2	54	25 : 29	20	9	11	0
8.2	49	24 : 25	20	10	8	2
17.7a	28	9 : 19	20	4	11	5

a: The sex ratio obtained from gonadal histology differed significantly at $p < 0.001$.

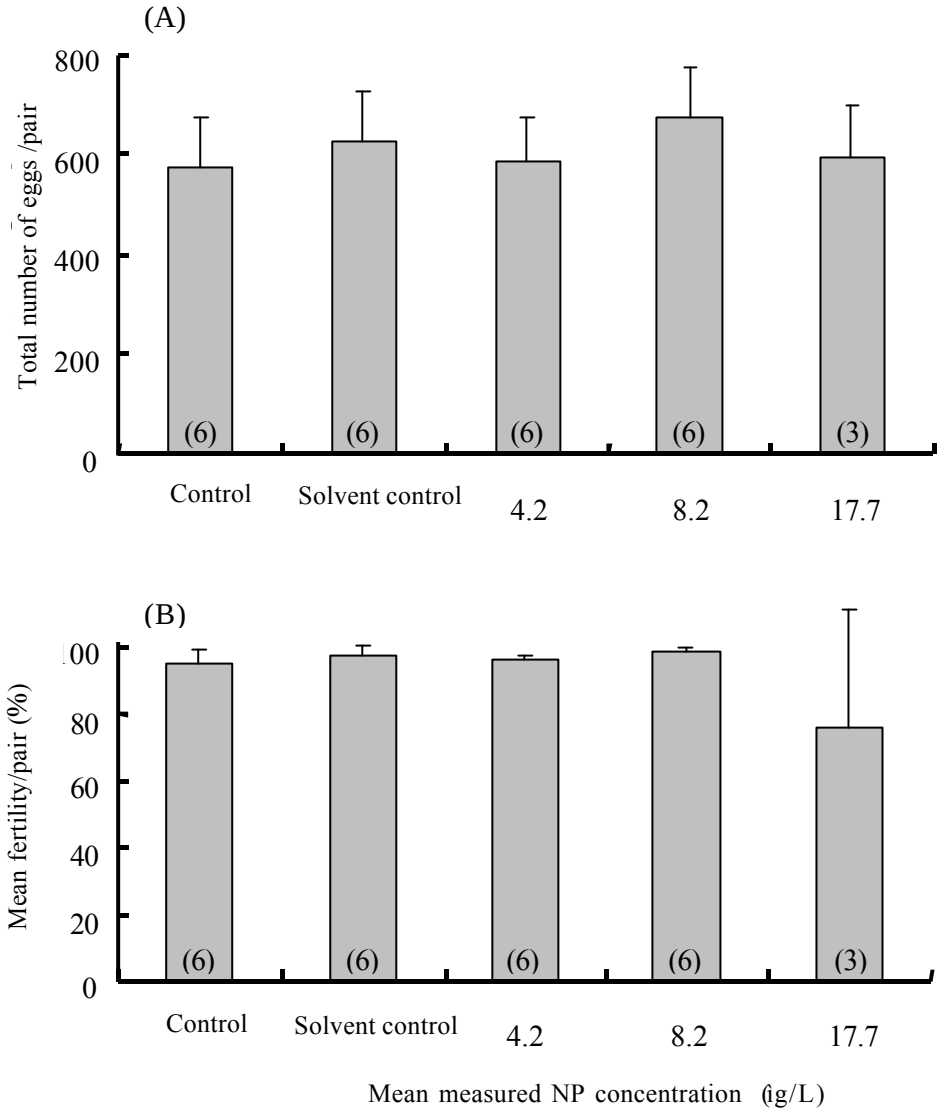

Fig. 5 Total number of eggs spawned by each pair from 71- to 104-day psothatch (A), and mean fertility per pair (B). Data is shown as mean ±standard deviation. The number of pairs in each treatment

1-3. Hazard assessment of 4-Nonylphenol on fish

Within the literature information of 1972-2000 obtained through TOXLINE, etc., in-water nonylphenol concentrations suspected to have endocrine disrupting effects on fish in the reported test results, whose reliability was confirmed, were 1.6ìg/L where abnormality in testis tissue of fathead minnow was observed in electron microscopic examination, 10ìg/L where vitellogenin mRNA was induced in liver of juvenile rainbow trout, 20.3ìg/L where vitellogenin was produced in plasma of mature male rainbow trout (the threshold value was estimated at 10ìg/L in the report), etc.

Within the *in-vitro* test results, nonylphenol showed the relative strength of

binding to estrogen receptor at 1/10, as compared with E_2, in medaka receptor binding assay, and at 1/200 in mummichog receptor binding assay, and the transcription activating power at a several-hundredth, as compared with E_2, in medaka reporter gene assay. Although, it was reported that nonylphenol's binding affinity was in the range of 1/2,000 to 1/3,000, as compared with E_2, in the test of binding to estrogenic receptor using Atlantic croaker, but the test concerned examined responses not in the receptor alone, but also in the cell from which its cytosol was extracted along with its surrounding cells. So the reported data is deemed lacking in reliability, as compared with MOE's test series examining genuine binding to receptor.

Within the screening results, in male medaka vitellogenin assay, significant production of vitellogenin was observed at concentration of 22.5 ìg/L in water (NB.:not observed at ≦12.8 ìg/L), and in medaka partial life cycle test, feminization of males in secondary sexual character at concentration of 23.5 ìg/L in water, and appearance of testis-ovas and production of vitellogenin at concentration of 11.6 ìg/L in water were observed significantly (NB.:not observed at ≦6.08 ìg/L).

Further, in medaka full life cycle test, abnormality in sex differentiation of males, decrease in fertilization rate, etc. were observed at concentration of 17.7 ìg/L in water, and testis-ovas not observed in the first generation were observed in the second generation at 8.2 ìg/L (NB.: not observed at 4.2 ìg/L).

As for nonylphenol, it has been reported in the past that vitellogenin was induced at low concentrations, indicating suspected endocrine disrupting activity to fish. However, there are many unknown points concerning vitellogenin, including the fact that while it is deemed peculiar to female, it was also observed in male fish not exposed to nonylphenol. As a result, vitellogenin was only used as a biomarker in screening techniques, and could not become an index to judge the existence or extent of endocrine disrupting activities. Under these circumstances, it may be safely said that this is the first evidence in the world to show, with regard to the suspected endocrine disrupting effects of nonylphenol, that morphogenetic abnormality such as testis-ova was observed at low concentrations in the test using medaka, sex ratio of which hardly changes despite environmental change. In supporting this, it was proved in *in-vitro* tests that nonylphenol has strong binding affinity to estrogen receptor and strong estrogenic effects on fish, though varied widely by fish species. As mentioned above, it was strongly supposed that nonylphenol has strong endocrine disrupting effects on fish.

Typical gonadal sections of testis and testis-ova are shown in Fig.6. Some of sections regarding normal testis and ovary development and secondary sexual characteristics from embryo to almost matured adult stage will be available from:

http://www.nies.go.jp/edc/edcdb/imdex_e.html

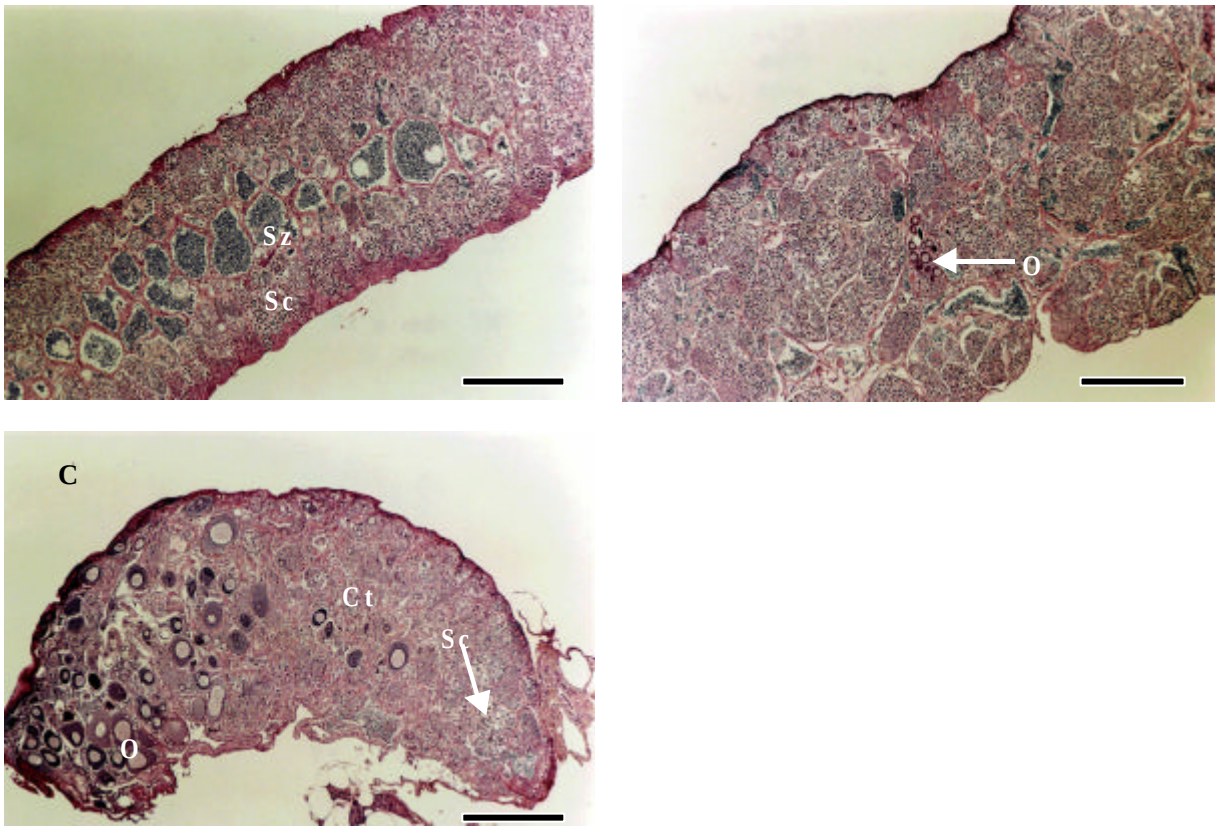

Figure 6. Gonadal sections (4 µm) from medaka at the end of exposure (60 d posthatch) in the partial life-cycle test (PLC) of NP treatment, stained with hematoxylin and eosin. Each bar shows 400µm length. (A) Testis of a control male in the NP exposure experiment, showing normal spermatogenesis: spermatocytes and spermatozoa. (B) Testis-ova of a male exposed to 23.5 µg/L NP. Oocytes appear in clusters within the testicular tissue. (C) More progressed testis-ova exposed to 44.7 µg/L NP. More than half of the area is composed of oocytes, accompanying abnormal connective tissue and small testicular tissues interspersed with a few spermatocytes.

Ct, connective tissue; O, oocytes; Sc, spermatocytes; Sz, spermatozoa.

2. Tributyltin

2-1. *In vitro* assays

2-1-1. Competitive binding assay to medaka estrogen receptor

The binding affinities of TBTCl to medaka (*Oryzias latipes*) and human estrogen receptors were measured by competitive binding assay using the ligand binding domain of these estrogen receptors(á) fused with glutathione S-transferase (GST), expressed in E. coli, and radiolabelled estradiol as a ligand. At TBTCl concentration of more than 10-6 M, the release of the ligand from estrogen receptor depending on the concentration of TBTCl was observed with both receptors. There is not any difference in release curves between medaka and human estrogen receptors. These results, however, do not indicate that TBTCl replace the ligand specifically bound to estrogen receptor because there is the possibility that the ligand could be released by denaturing of estrogen receptor with TBTCl due to its strong protein denaturing ability. Then the denaturing of estrogen receptor was measured as a function of TBTCl by monitoring the enzyme activity of GST fused to the receptor, which is expected to be denatured with TBTCl in the same manner as the receptors. The similar relationship between the enzymatic activity and TBTCl concentration as the release of ligand was observed though the decreases of the enzyme activity begin at slight lower concentration than the release of ligand. These results strongly suggest that the release of ligand from estrogen receptors is caused by denaturing of the receptors by TBTCl（Fig7 and Table6）.

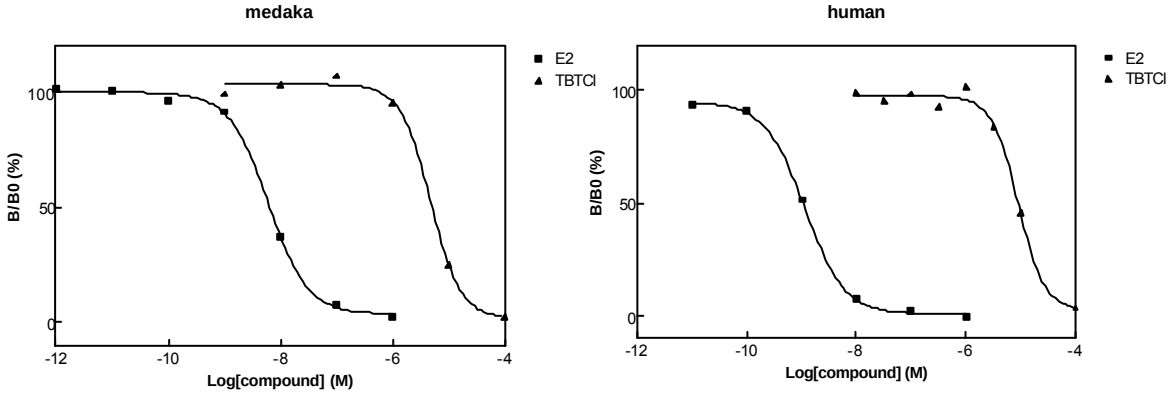

Fig. 7 Elimination of [^{3}H]-estradiol from estrogen receptor by estradiol and TBTCl (left: medaka; right: human

Table6. Relative binding strength of TBTCl obtained from [3H]-estradiol elimination curves

Species	Relative binding strength (%)	
	Estradiol	TBTCl
Medaka	100	0.13
Human	100	0.013

2-1-2. Reporter gene assay

The expression and reporter plasmids were transiently co-transfected to HeLa cell exposed with TBTCl, and after incubation overnight the luciferase activity induced by transcriptional activation by TBTCl bound estrogen receptor was measured as a function of TBTCl concentration. No enhancement of the luciferase activity was observed over 10^{-12} to 10^{-3} M of TBTCl, but the activity decreased from the basal value at more than10nM. The decreasing curve of luciferase activity from basal activity is similar to decreasing one of GST activity, which is the indication of denaturing of estrogen receptor. These results supported the finding that TBTCl denature proteins in cell including estrogen receptor and consequently inhibit of cell activity at these concentrations.

In conclusion, no data indicating that TBTCl bind specifically to estrogen receptor is obtained from competitive binding and reporter gene assays.

2-2. *In vivo* studies using medaka

2-2-1. Medaka vitellogenin assay

This study was conducted to assess the effects of tributyltin chloride (TBTCl) on vitellogenin (precursor of egg yoke protein) synthesis in medaka. About 3-month-old medaka (respectively 10 females and males/treatment) were exposed to TBTCl at the concentrations of 117, 269, 606, 1,640 and 4,000ng/L (mean measured concentrations) under flow-through conditions for 21days. 17â-estradiol (E2, 100ng/L) was tested as positive control. Daily observation was made to examine mortality and abnormal behavior and appearance during the exposure period. At the end of exposure, the livers of fish were removed, and vitellogenin concentration in each liver was measured.

Neither death nor particular symptom was observed during the exposure period. At the end of exposure, the hepatosomatic index (HSI) of male fish exposed to ≥ 269 ng/L was significantly higher than that in the controls. As for vitellogenin

concentration, however, no statistically significant change was observed in both males and females in any treatment, as compared with that in the controls (Fig.8).

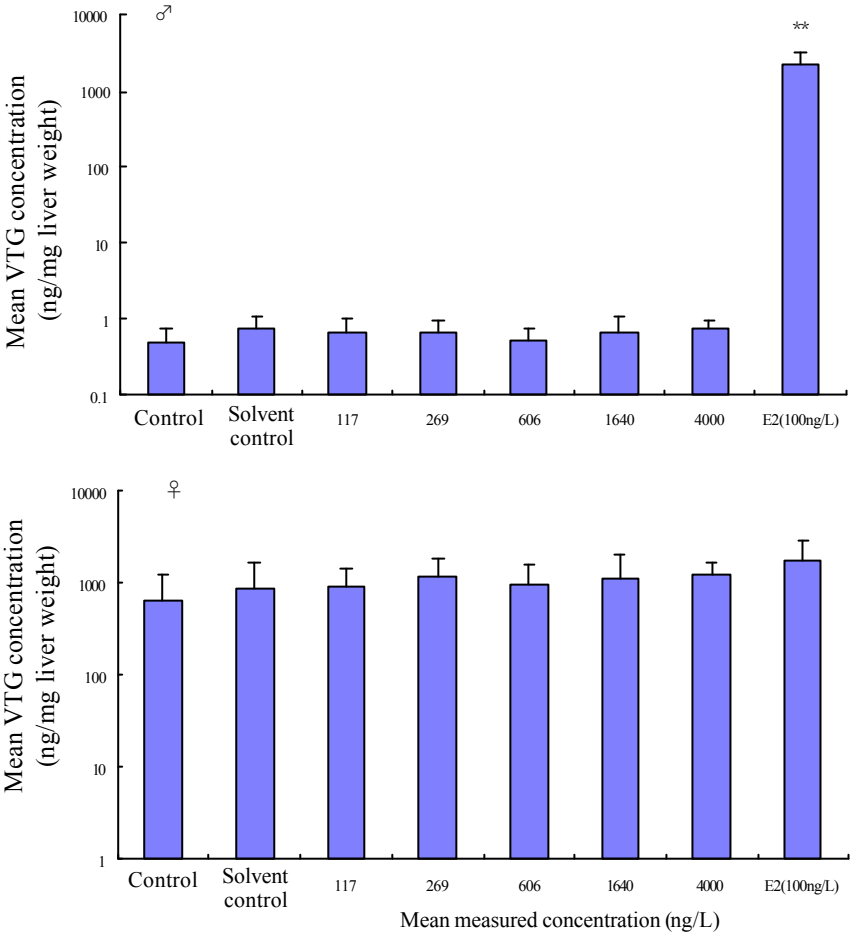

Fig. 8 Vitellogenin (VTG) concentrations in the livers of male and female medaka (*Oryzias latipes*) in the vitellogenin assay. Data is shown as mean ± standard deviation. ** denotes a significant difference at $p < 0.01$.

2-2-2. Medaka partial life test

This test was conducted to evaluate endocrine disrupting effects of tributyltin chloride (TBTCl) on sexual differentiation of medaka. Medaka (60 eggs/treatment) was exposed to TBTCl at the concentrations of 20.1, 64.1, 205, 594 and 1,650ng/L (mean measured concentrations) under flow-through conditions from fertilized eggs to 61-day posthatch. No significant effects were observed on hatching of embryos at the concentrations tested. However, posthatch mortality in the highest treatment (1,650ng/L) increased markedly, and the cumulative mortality at 61-day posthatch was

significantly higher than the control mortality. The growth of fish at 61-day posthatch were suppressed with increasing TBTCl concentrations, resulting in significant differences in the total length at 594 and 1,650ng/L, and in the body weight at 1,650ng/L. These results suggested that TBTCl would have lethal toxicity or growth-inhibitory effects on medaka larvae and juveniles at ≥ 594ng/L. Based on an examination of the secondary sex characteristics of the surviving fish at 61-day posthatch, there were no significant differences in the sex ratio in any treatments, although more females than males were identified in the 594ng/L and 1,650ng/L treatments. Gonadal histology showed that histological abnormalities, such as hermaphrodism, were not observed in any treatments (Table 7). The HSI of fish at 61-day posthatch was increased in 594ng/L and 1,650ng/L treatments, suggesting that TBTCl exerts hepatotoxicty at these concentrations. The hepatic vitellogenin concentrations in males at the end of exposure were significantly increased in all treatments relative to the solvent controls, but not to the controls. And there was no clear concentration-response relationship between the vitellogenin concentrations and the TBTCl treatments. It was not possible, therefore, to conclude that there was a clear vitellogenin induction in male medaka (Fig.9). The vitellogenin concentration in females did not show significant differences in any treatments.

As mentioned above, it was suggested that TBTCl would have chronically lethal toxicity or growth inhibitory effects on medaka at ≥ 594ng/L TBTCl. In this test, however, it was not observed that TBTCl affected sexual differentiation of medaka by its endocrine disrupting effects.

Table 7. Sex ratios as determined by gross examination of secondary sex characteristics of medaka (*Oryzias latipes*) at 61- day posthatch and by their gonadal histology.

TBTCl concentration* (ng/L)	Secondary sex characteristics		Gonadal histology		
	N	Sex ratio (male:female	*N*	Number of fish	
				Testis	Ovary
Control	50	23 : 27	20	12	8
Solvent control	53	28 : 25	20	14	6
20.1	53	26 : 27	20	11	9
64.1	50	23 : 27	20	7	13
205	51	25 : 26	20	10	10
594	53	22 : 31	20	7	13
1,650	33	13 : 20	20	8	12

* Shown in mean measured concentrations.

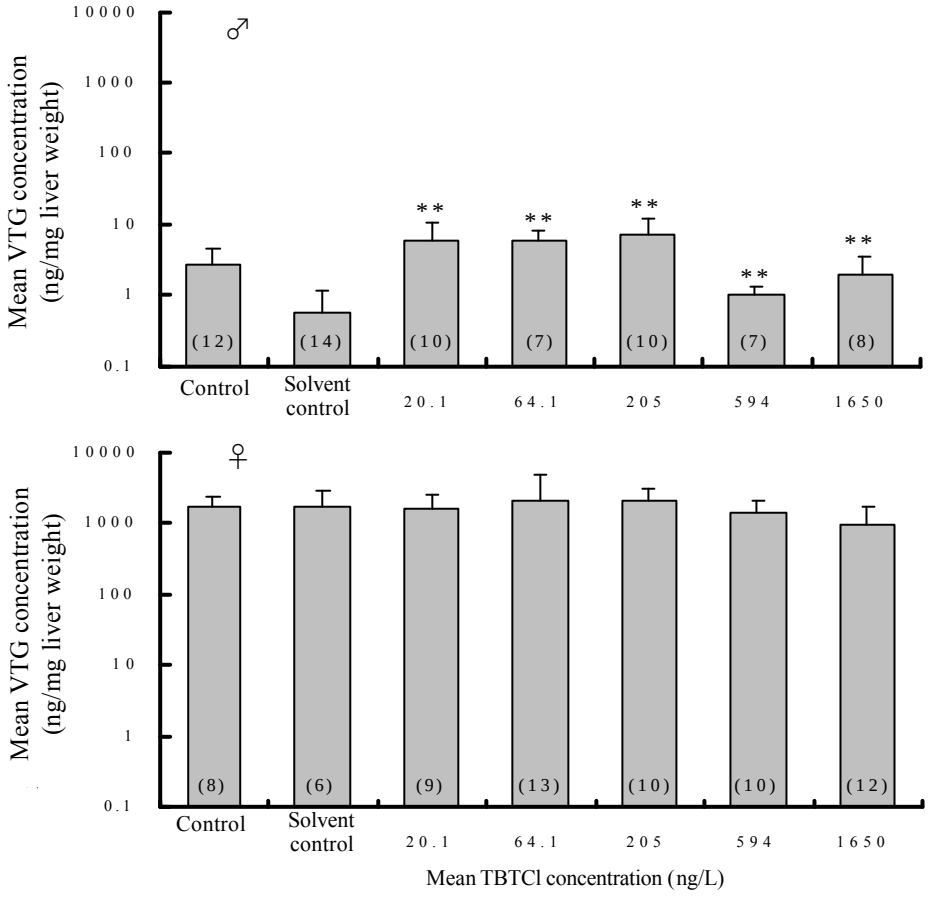

Fig. 9 Vitellogenin (VTG) concentrations in the livers of medaka (*Oryzias latipes*) at 61-day posthatch in each treatment. Data is shown as mean±standard deviation. Numbers in parentheses refer to number of fish. ** denotes a significant difference relative to the solvent controls at $p < 0.01$.

2-3. Effects on fish

Of the literature information of 1972-2000 obtained through TOXLINE, etc., no report was obtained on the *in vitro* test on estrogenic or androgenic effects of fish. In animal tests, effects on sheepshead minnow were examined, but in any exposure group, no effect on reproduction was observed [1]. On the other hand, though reliability assessment was not conducted, a report was obtained that TBT had an effect on sex differentiation of flounders, causing sex reversal of hereditary total females [2].

Of MOE's *in vitro* test results, the medaka receptor binding assay showed that the binding was not high because the relative binding strength to estrogen receptor was about 1/1000, as compared with E_2. It was also strongly indicated that the binding could possibly be affected by TBTCl's denaturing activity.

Of MOE's screening results, in the male medaka vitellogenin assay, significant change was not observed at any concentration.

Also, in the medaka partial life-cycle test, an increase in female rate was observed at TBTCl concentration of 594ng/L or higher, but without any significant difference. Any physiological abnormality, such as hermaphrodite, was not observed, either. As for male medaka vitellogenin, a statistically significant difference was observed at all TBTCl concentration sectors against auxiliary agent sector, but not against control sector. And the change in vitellogenin concentration did not show any clear dependence on TBTCl concentration. So, it was not concluded that any clear vitellogenin induction occurred in male bodies.

As mentioned above, from the results of MOE's various tests using medaka and from the literature information on which reliability assessment was carried out, any clear result was not obtained that tributyltin (TBT) compounds had endocrine disrupting effect on fish. However, there was a report that sex reversal was caused in flounders[2], though it is not known whether the reversal was due to endocrine disruption or not. Further, it is not known yet whether such a sex reversal was a response peculiar to flounders liable to reverse sex in response to water temperature change or stress, whether it was due to difference in sensitivity among fish species, or whether it was due to difference in exposure methods (water exposure, feed exposure) or resultant exposure concentration, etc. The report also assumed that the masculinization was caused by the TBT activity as aromatase inhibitor, but the world's effort to elucidate the effect on aromatase was just started. So it is necessary to accumulate further scientific data on various fish species, including verification of reproducibility of sex reversal as observed in flounders.

Reference

1. Manning,C.S., Lytle,T.F.,Walker,W.W. and Lytle,J.S.(1999) Life-cycle toxicity of bis(tributyltin) oxide to the sheepshead minnow (*Cyprinodon variegatus*). Arch.Environ.Contam.Toxicol.,37, 258-266.

2. Shimazaki, Y., Kitano, T., Oshima, Y., Imada, N. and Honjo, T. (2000) Masculinization of flounders by tributyltin, Collected summaries of lectures at the 3rd symposium of Japan Society of Endocrine Disrupter Research, A-3-1, 65.

Results of Assay and Tests in Evaluation of the Endocrine Disrupting Activities in Fish(*Medaka*)

4-*tert*-Octylphenol

1 Vitellogenin Assay

Table 1. Results

Treatment (ìg/L)	Mortality (%)	Hepatosomatic index (%) male	Hepatosomatic index (%) female	Vitellogenin (ng/mg liver) male	Vitellogenin (ng/mg liver) female
Control	0	1.9 ± 1.0	4.8 ± 1.0	3.6± 3.5	1,500 ± 320
Solvent control	0	1.8 ± 0.9	4.0 ± 1.1	1.4± 1.2	1,800 ± 1,300
12.7	0	2.0 ± 0.6	4.4 ± 0.8	1.9± 1.2	1,800 ± 540
27.8	0	1.8 ± 0.3	4.0 ± 0.6	3.6± 4.4	1,900 ± 510
64.1	0	2.2 ± 0.8	4.3 ± 1.1	190 ± 370**	1,500 ± 400
129	6.3	2.6 ± 0.3	3.8 ± 0.9	2,300 ± 1,100**	3,000 ± 2,900
296	0	2.8 ± 0.6	4.2 ± 0.9	6,100 ± 1,800**	3,300 ± 1,900**

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

2 Partial Life---Cycle Test

Table 2-A. Results

Treatment (ìg/L)	Hatchability (%)	Time to hatching (day)	Mortality (%)	Total length (mm)	Body weight (mg)
Control	98 ± 3.3	9.1 ± 0.3	5.0 ± 3.3	26.2± 2.1	188 ± 35
Solvent control	98 ± 3.3	9.0 ± 0.1	5.0 ± 6.4	26.2± 2.0	157 ± 35
6.94	95 ± 6.4	8.9 ± 0.1	3.6 ± 4.2	26.6± 1.8	163 ± 37
11.4	98 ± 3.3	9.0 ± 0	5.1 ± 6.4	26.6± 1.5	169 ± 34
23.7	100 ± 0	8.9 ± 0.1	20 ± 12*	27.3 1.6**	187 ± 39##
48.1	95 ± 6.4	9.0 ± 0	1.9 ± 3.9	26.1± 1.7	167 ± 34
94.0	97 ± 6.7	9.0 ± 0	5.5 ± 7.3	25.5± 2.1	159 ± 39

Table 2-B. Results (continued)

Treatment (ìg/L)	Gonadosomatic index (%) male	Gonadosomatic index (%) female	No. of fish	No. of males with testis—ova/No. of males	Hepatosomatic index (%) male	Hepatosomatic index (%) female	Vitellogenin (ng/mg liver) male	Vitellogenin (ng/mg liver) female
Control	0.47 ±0.2	1.8 ± 1.8	20	0/10	2.2 ± 0.6	3.1 ± 0.5	1.9 ± 1.8	810± 600
Solvent control	0.85 ±0.9	2.9 ± 2.9	20	0/9	2.1 ± 0.7	3.5 ± 0.6	0.83 ±0.7	810± 770
6.94	0.78 ±0.3	4.5 ± 2.9	20	0/10	2.8 ± 0.6*	3.8 ± 0.8	5.4 ± 4.5	1,600± 1,000
11.4	0.88 ±0.4	4.4 ±3.0	20	1/9	2.7 ± 0.5	3.3 ± 0.8	13 ± 9.4**	2,300± 1,700
23.7	0.71 ±0.3	3.2 ±3.3	20	2/10 *	2.8 ± 0.8*	4.1 ± 0.7*	17 ± 19**	1,700± 1,300
48.1	0.64 ±0.2	2.8 ±2.4	20	3/10 *	2.4 ± 0.7	3.9 ± 0.7	140 ± 190**	3,600± 1,400**
94.0	0.39 ±0.4	0.60 ±0.5**	20	5/10 **	3.3 ± 0.6**	4.0 ± 0.9	500 ± 880**	4,000± 790**

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)
Statistically significant differences from solvent control group(##indicates $p<0.01$, #indicates $p<0.05$)

3.Full Life---Cycle Test

Table 3-A. F_0 generation

Treatment (ìg/L)	Hatchability (%)	Time to hatching (day)	Mortality (%)	Total length (mm)	Body weight (mg)	No. of fish	No. of males with testis—ova/No. of males
Control	100	8.5 ± 0.3	1.7	30.5 ± 1.3	269 ± 32	20	0/8
Solvent control	97 ± 3.9	8.4 ± 0.2	6.7	31.6 ± 1.4	307 ± 43	20	0/9
1.68	88 ± 11	8.1 ± 0.2	8.4	31.7 ± 1.8	310 ± 62	20	0/10
4.27	92 ± 8.4	8.2 ± 0.4	5.8	31.5 ± 1.2	298 ± 35	20	0/10
9.92	97 ± 6.7	8.4 ± 0.1	12	32.0 ± 1.2	301 ± 42	20	1/10
30.4	88 ± 6.4	8.3 ± 0.4	11	32.1 ± 1.5	322 ± 50	20	5/7 **
82.3	92 ± 8.4	8.2 ± 0.1	5.8	31.7 ± 1.5	310 ± 44	20	7/8 **

Table 3-B. F_0 generation (continued)

Treatment (ìg/L)	No. of eggs	Fertility (%)	Gonadosomatic index (%)		Hepatosomatic index (%)		Vitellogenin (ng/mg liver)	
			male	female	male	female	male	female
Control	653 ± 89	97 ± 3.3	0.81 ± 0.1	7.5 ± 1.5	1.7 ± 0.6	5.5 ± 1.8	10 ± 15	1,600 ± 740
Solvent control	500 ± 170	90 ± 15	0.65 ± 0.2	7.7 ± 1.7	1.6 ± 0.4	4.1 ± 0.7	8.6 ± 8.8	1,600 ± 1,300
1.68	659 ± 130	97 ± 1.2	0.86 ± 0.2	7.6 ± 1.5	1.4 ± 0.5	4.2 ± 1.5	8.5 ± 8.8	1,700 ± 780
4.27	667 ± 60	98 ± 2.1	0.98 ± 0.2	8.0 ± 0.7	1.4 ± 0.2	3.8 ± 0.4	16 ± 10	2,100 ± 1,100
9.92	631 ± 80	93 ± 7.4	0.93 ± 0.2	8.3 ± 1.2	1.8 ± 0.8	3.5 ± 0.7	290 ± 640**	2,600 ± 2,400
30.4	520 ± 150	92 ± 8.0	0.92 ± 0.3	7.8 ± 1.9	1.9 ± 0.1	4.5 ± 0.8	630 ± 850**	4,900 ± 2,600**
82.3	45 ± 87**	35 ± 36*	1.0 ± 0.3	8.2 ± 3.8	2.6 ± 0.7**	3.9 ± 0.7	2,800 ± 2,800**	11,000 ± 6,700**

Table 3-C. F_1 generation

Treatment (ìg/L)	Hatchability (%)	Time to hatching (day)	Mortality (%)	Total length (mm)	Body weight (mg)	No. of fish	No. of males with testis—ova/No. of males
Control	94 ± 7.6	9.7 ± 0.4	1. 7	28.7 ± 1.6	252 ± 45	20	0/11
Solvent control	80 ± 29	9.4 ± 0.6	6.7	28.9 ± 1.7	253 ± 41	20	0/12
1.68	90 ± 14	9.2 ± 0.4	6.7	28.2 ± 1.7	242 ± 39	20	0/14
4.27	92 ± 7.8	9.4 ± 0.5	8.3	28.7 ± 1.7	243 ± 37	20	0/11
9.92	96 ± 6.8	9.5 ± 0.6	0	28.3 ± 2.1	243 ± 27	20	0/8
30.4	97 ± 7.4	9.5 ± 0.5	0	28.7 ± 1.1	243 ± 30	20	4/8 **
82.3	51 ± 49	9.6 ± 0.3	6.1	28.8 ± 1.0	252 ± 28	20	10/15 **

Table 3-D. F_1 generation (continued)

Treatment (ìg/L)	Vitellogenin (ng/mg liver)	
	male	female
Control	3.1 ± 2.6	1,700 ± 1,000
Solvent control	4.3 ± 5.0	1,500 ± 1,000
1.68	3.1 ± 4.4	990 ± 920
4.27	6.5 ± 19	2,300 ± 1,100
9.92	24 ± 22**	3,200 ± 1,200**
30.4	42 ± 29**	4,300 ± 2,000**
82.3	22 ± 22*	6,200 ± 540**

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

Di-n-butyl phthalate

1.Vitellogenin Assay

Table 1. Results

Treatment (ìg/L)	Mortality (%)	Hepatosomatic index (%) male	Hepatosomatic index (%) female	Vitellogenin (ng/mg liver) male	Vitellogenin (ng/mg liver) female
Control	0	1.9 ± 0.4	4.6 ± 0.9	0.5± 0.1	1,200 ± 580
Solvent control	0	1.8 ± 0.6	4.1 ± 1.2	0.8± 0.8	1,200 ± 580
24.4	0	2.0 ± 0.6	4.1 ± 1.2	0.5± 0.2	950 ± 720
55.3	0	2.1 ± 1.0	4.4 ± 0.8	0.7± 0.3	1,200 ± 560
133	0	2.3 ± 0.7	4.5 ± 1.1	0.7± 0.5	660 ± 610
328	5	2.5 ± 0.6*	5.6 ± 1.8	0.4 ± 0.1	790 ± 780
822	0	2.8 ± 0.6**	4.3 ± 0.9	0.4± 0.1	1,100 ± 880

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

2.Partial Life---Cycle Test

Table 2-A. Results

Treatment (ìg/L)	Hatchability (%)	Time to hatching (day)	Mortality (%)	Total length (mm)	Body weight (mg)
Control	97 ± 3.9	10 ± 0.1	8.6 ± 6.6	29.5± 1.4	256 ± 42
Solvent control	97 ± 3.9	10 ± 0.2	11 ± 4.4	29.6± 1.3	256 ± 41
7.09	95 ± 6.4	9.8 ± 0.1	12 ± 8.6	29.8± 1.5	266 ± 50
21.9	87 ± 14	9.8 ± 0.2	8.9 ± 10	29.5± 1.4	259 ± 46
72.8	97 ± 3.9	9.9 ± 0.1	21 ± 5.1*	30.1± 1.5	269 ± 38
235	100	10 ± 0.3	48 ± 18*	29.6± 1.7	269 ± 48
850	8.3 ± 10*	16 ± 0.7	100	---	---

---indicates 100% mortality

Table 2-B. Results (continued)

Treatment (ìg/L)	Gonadosomatic index (%) male	Gonadosomatic index (%) female	No. of fish	No. of males with testis-ova/No. of males	Hepatosomatic index (%) male	Hepatosomatic index (%) female	Vitellogenin (ng/mg liver) male	Vitellogenin (ng/mg liver) female
Control	0.64 ±0.2	3.9 ± 2.5	20	0/10	3.4 ± 0.9	4.1 ± 0.6	2.4 ± 3.8	1,200± 950
Solvent control	0.68 ±0.2	2.5 ± 2.7	20	0/12	3.2 ± 1.0	5.0 ± 1.4	0.83 ± 1.6	760± 1,200
7.09	0.61 ±0.2	3.9 ± 3.4	20	1/11	3.6 ± 0.9	5.6 ± 0.6*	3.5 ± 3.3	1,400± 1,200
21.9	0.63 ±0.3	4.5 ±4.0	20	0/12	3.5 ± 1.2	4.4 ± 1.0	1.2 ± 2.6	1,400± 840
72.8	0.73 ±0.3	4.6 ± 3.8	20	2/12 *	3.2 ± 1.1	4.3 ± 0.8	4.1 ± 8.0	1,200± 1,300
235	0.63 ±0.3	2.8 ± 3.6	20	0/9	3.4 ± 0.8	4.2 ± 1.0	3.7 ± 5.6	360± 730
850	—	—	—	—	—	—	—	—

--- ;indicates 100% mortality

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

3.Full Life - Cycle Test

Table 3-A. F_0 generation

Treatment (ìg/L)	Hatchability (%)	Time to hatching (day)	Mortality (%)	Total length (mm)	Body weight (mg)	No. of fish	No. of males with testis-ova/No. of males
Control	92 ± 8.4	9.9 ± 0.3	13	29.1 ± 1.4	236 ± 44	20	0/8
Solvent control	95 ± 6.4	10 ± 0.4	18	29.6 ± 1.2	245 ± 35	20	0/9
2.61	98 ± 3.3	9.8 ± 0.2	12	28.6 ± 1.5	218 ± 39	20	1/8
7.52	97 ± 3.8	9.8 ± 0.2	16	29.3 ± 1.3	239 ± 34	20	0/11
23.9	95 ± 6.4	10 ± 0.3	5.6	29.1 ± 1.4	233 ± 38	20	0/6
74.5	95 ± 6.4	10 ± 0.1	24	30.1 ± 1.7	259 ± 49	20	1/9
233	98 ± 3.3	10 ± 0.2	15.	28.6 ± 1.8	226 ± 50	20	2/8 *

Table 3-B. F_0 generation(continued)

Treatment (ìg/L)	No. of eggs	Fertility (%)	Gonadosomatic index (%)		Hepatosomatic index (%)		Vitellogenin (ng/mg liver)	
			male	female	male	female	male	female
Control	560 ± 210	94 ± 6.5	0.78 ± 0.2	9.3 ± 1.6	1.3 ± 0.4	4.2 ± 0.8	14.0 ± 30	2,000 ± 2,000
Solvent control	625 ± 130	87 ± 26	0.89 ± 0.2	8.1 ± 0.9	1.5 ± 0.3	4.2 ± 0.7	7.0 ± 6.3	1,600 ± 950
2.61	602 ± 110	96 ± 5.6	0.86 ± 0.2	7.5 ± 0.7	1.4 ± 0.3	4.0 ± 0.7	9.9 ± 9.5	1,500 ± 890
7.52	668 ± 100	94 ± 8.9	0.92 ± 0.2	8.0 ± 0.9	1.4 ± 0.2	4.0 ± 0.3	15 ± 9.4	1,400 ± 330
23.9	543 ± 110	94 ± 3.1	1.1 ± 0.1*	9.0 ± 0.5	1.3 ± 0.4	4.5 ± 0.6	8.1 ± 7.4	1,800 ± 470
74.5	554 ± 180	97 ± 1.6	0.92 ± 0.2	7.8 ± 1.1	1.6 ± 0.4	3.8 ± 1.3	13 ± 13	1,700 ± 520
233	539 ± 240	91 ± 11	0.97 ± 0.3	9.4 ± 2.6	1.8 ± 0.2	4.4 ± 1.0	4.6 ± 4.0	2,100 ± 2,200

Table 3-C. F_1 generation

Treatment (ìg/L)	Hatchability (%)	Time to hatching (day)	Mortality (%)	Total length (mm)	Body weight (mg)	No. of fish	No. of males with testis-ova/No. of males
Control	87 ± 8.9	9.4 ± 0.6	0	30.7 ± 1.2	276 ± 39	20	0/9
Solvent control	85 ± 11	9.4 ± 0.5	0	30.5 ± 1.4	281 ± 39	20	0/7
2.61	89 ± 8.8	9.1 ± 0.6	0	30.8 ± 1.2	274 ± 34	20	2/10
7.52	94 ± 6.4**	9.4 ± 0.5	1.7	31.7 ± 1.1**	297 ± 41*	20	2/13 *
23.9	72 ± 21	8.6 ± 1.1	1.7	30.8 ± 1.3	283 ± 33	20	1/11
74.5	90 ± 12	9.8 ± 0.4*	0	30.8 ± 1.3	290 ± 31	20	1/14
233	94 ± 6.6*	11 ± 1.2**	3.3	30.2 ± 1.2	292 ± 39	20	0/9

Table 3-D. F_1 generation (continued)

Treatment (ìg/L)	Vitellogenin(ng/mg liver)	
	male	female
Control	0.8 ± 1.1	440 ± 720
Solvent control	ND	470 ± 1,000
2.61	3.8 ± 5.0*	1,700 ± 820**
7.52	9.1 ± 8.5**	1,600 ± 1,100*
23.9	14 ± 29	1,200 ± 580**
74.5	3.3 ± 2.7**	850 ± 790
233	2.5 ± 3.0	730 ± 570

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

Di-(2-ethylhexyl) phthalate

1.Vitellogenin Assay

Table 1. Results

Treatment (ìg/L)	Vitellogenin (ng/mg liver)		Hepatosomatic index (%)	
	14-d	21-d	14-d	21-d
Control	ND	0.53±0.13	1.56±0.27	1.44±0.21
Solvent control	0.55±0.21	ND	1.50±0.27	1.46±0.24
19	0.62±0.46	ND	1.46±0.33	1.59±0.19
43	ND	ND	1.74±0.27	1.33±0.28
96	0.58±0.31	ND	1.60±0.38	1.59±0.32
210	ND	ND	1.54±0.18	1.44±0.34
410	ND	ND	1.74±0.22	1.39±0.31

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

2.Partial Life - Cycle Test

Table 2-A. Results

Treatment (ìg/L)	Hatchability (%)	Time to hatching (day)	Mortality (%)	Total length (mm)	Body weight (mg)
Control	93 ± 7.7	8.8 ± 0.5	0.0 ± 0.0	30.3 ± 1.5	271 ± 43
Solvent control	98 ± 3.3	9.2 ± 0.2	1.9 ± 3.9	30.4 ± 1.6	275 ± 52
11.0	93 ± 0	9.0 ± 0.3	1.8 ± 3.6	30.7 ± 1.5	290 ± 45
28.4	100 ± 0	9.1 ± 0.1	0.0 ± 0.0	30.1 ± 2.0	270 ± 58
73.4	95 ± 10	9.1 ± 0.1	0.0 ± 0.0	30.5 ± 1.6	263 ± 49
186	95 ± 6.4	9.0 ± 0.1	1.8 ± 3.6	30.2 ± 2.0	261 ± 51
446	95 ± 6.8	9.0 ± 0.2	2.1 ± 4.2	30.3 ± 2.0	264 ± 48

Table 2-B. Results (continued)

Treatment (ìg/L)	Gonadosomatic index (%)		No. of fish	No. of males with testis—ova / No. of males	Hepatosomatic index (%)		Vitellogenin (ng/mg liver)	
	male	female			male	female	male	female
Control	0.78 ± 0.21	5.0 ± 2.7	20	0/13	2.2 ± 0.7	3.7 ± 0.4	1.3 ± 1.4	1,100± 730
Solvent control	0.71 ± 0.23	6.2 ± 3.6	20	0/10	2.0 ± 0.2	3.9 ± 0.4	2.8 ± 3.6	1,600± 1,000
11.0	0.82 ± 0.27	3.8 ± 2.6	20	0/12	1.8 ± 0.6	3.9± 1.3	2.5 ± 4.1	1,100± 890
28.4	0.97 ± 0.40	4.3 ± 2.9	20	0/10	1.6 ± 0.7	3.5 ± 0.5	3.5 ± 5.4	1,500± 920
73.4	0.83 ± 0.26	5.2 ± 3.4	20	1/11	2.6 ± 0.9	3.7 ± 0.4	0.4 ± 0.4	1,500± 1,100
186	0.76 ± 0.26	6.4 ± 3.9	20	0/10	2.3 ± 0.6	4.2 ± 0.9	0.5 ± 0.5	1,700± 1,100
446	0.86 ± 0.37	6.0 ± 3.3	20	0/12	2.3 ± 0.7	3.9 ± 0.5	4.3 ± 9.3	1,200± 570

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

Di-cyclohexyl phthalate

1.Vitellogenin Assay

Table 1. Results

Treatment (ìg/L)	Vitellogenin (ng/mg liver)		Hepatosomatic index (%)	
	14-d	21-d	14-d	21-d
Control	0.55±0.21	ND	1.55±0.13	1.42±0.20
Solvent control	ND	ND	1.67±0.21	1.40±0.26
18	ND	ND	1.58±0.28	1.39±0.18
38	0.53±0.13	ND	1.56±0.23	1.61±0.22*
87	ND	ND	1.88±0.37*	1.56±0.21
190	ND	ND	1.84±0.26*	1.50±0.20
390	ND	ND	2.04±0.37**	1.55±0.39

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

2 . Partial Life Cycle Test

Table 2-A. Results

Treatment (ìg/L)	Hatchability (%)	Time to hatching (day)	Mortality (%)	Total length (mm)	Body weight (mg)
Control	98 ± 3.3	9.7 ± 0.2	0 ± 0	28.0 ± 1.4	220 ± 36
Solvent control	92 ± 13	9.2 ± 0.3	3.3 ± 6.7	27.3 ± 2.8	250 ± 50
0.429	100 ± 0	9.1 ± 0.1	1.8 ± 3.6	28.8 ± 1.5**	225 ± 41#
1.41	93 ± 9.4	9.1 ± 0.1	7.6 ± 11	28.4 ± 2.3	241 ± 44
4.39	92 ± 8.4	9.1 ± 0.1	5.6 ±7.3	30.0 ± 1.6**	250 ± 47
13.3	100 ± 0	9.3 ± 0.4	0 ± 0	29.0 ± 1.7**	237 ± 45
35.8	90 ± 8.6	9.1 ± 0.1	13 ± 10	29.8 ± 1.8**	265 ± 48

Table 2-B. Results (continued)

Treatment (ìg/L)	Gonadosomatic index (%) male	female	No. of fish	No. of males with testis-ova / No. of males	Hepatosomatic index (%) male	female	Vitellogenin (ng/mg liver) male	female
Control	0.75 ± 0.2	4.3 ± 3.3	20	0/13	2.7 ± 0.7	3.6 ± 1.0	1.8 ± 2.4	1,600± 1,500
Solvent control	0.74 ± 0.2	5.2 ± 3.3	20	0/12	2.5 ± 0.4	4.0 ± 0.7	2.2 ± 2.4	1,800± 1,300
0.429	0.83 ± 0.2	5.5 ± 3.1	20	0/13	2.4 ± 0.4	3.6 ± 0.9	3.8 ± 3.4	2,100± 1,100
1.41	0.69 ± 0.2	2.9 ± 2.6	20	0/13	2.4 ± 0.6	3.0 ± 0.5	4.7 ± 4.7	1,600± 1,400
4.39	0.85 ± 0.3	5.8 ± 3.7	20	0/14	2.2 ± 0.6	3.6 ± 0.5	12 ± 16**	1,800± 660
13.3	0.76 ± 0.2	3.9 ± 2.8	20	0/11	2.1 ± 0.5	3.2 ± 0.7	1.3 ± 2.0	2,400± 1,900
35.8	1.1 ± 0.3**	5.9 ± 3.1	20	1/10	2.2 ± 0.9	3.7 ± 1.0	2.7 ± 2.1	2,900± 3,300

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)
Statistically significant differences from solvent control group(##indicates $p<0.01$, #indicates $p<0.05$)

Di-ethyl phthalate

1.Vitellogenin Assay

Table 1. Results

Treatment (ìg/L)	Vitellogenin (ng/mg liver)		Hepatosomatic index (%)	
	14-d	21-d	14-d	21-d
Control	4.4±1.1	1.3±0.1	2.05±0.11	1.65±0.13
8.1	2.1±0.2	2.3±0.6	1.95±0.19*	1.75±0.34
26.8	4.8± 1.5	1.8±0.5	1.87±0.13*	1.64±0.11
119.8	2.7± 0.8	2.2± 0.8	2.00±0.15*	2.41±0.76
355.8	2.4±0.4	1.0± 0.1	1.91±0.14*	1.61±0.10
1,053.3	2.5±0.7*	1.2± 0.2	1.98±0.11*	1.76±0.06

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

2. Partial Life - Cycle Test

Table 2-A. Results

Treatment (ìg/L)	Hatchability (%)	Time to hatching (day)	Mortality (%)	Total length (mm)	Body weight (mg)
Control	81	11.2± 0.3	8.6	20.8± 0.3	164.5± 6.6
0.6	80	12.3± 0.4	3.8	20.6± 0.2	158.1± 5.4
2.5	83	12.4 ± 0.4*	13.3	21.1± 0.2	167.7± 4.5
8.4	91	12.3± 0.5*	17.6	21.5± 0.2	167.8± 4.1
36.0	92	11.8± 0.3*	5.4	20.1± 0.2*	142.0± 3.8*
121.6	88	11.3± 0.3	2.3	20.3± 0.2	140.5± 3.9*

Table 2-B. Results (continued)

Treatment (ìg/L)	Gonadosomatic index (%)		No. of fish	No. of males with testis-ova / No. of males	Hepatosomatic index (%)		Vitellogenin (ng/mg liver)	
	male	female			male	female	male	female
Control	1.09±0.07	7.54±0.19	20	0/10	4.49±0.50	4.24±0.60	0.16 ± 0.05	255.7± 95.0
0.6	0.87±0.10	7.40±0.21	20	0/10	4.19±0.36	3.85±0.52	0.21 ± 0.08	160.0± 102.6
2.5	1.02±0.06	7.34±0.19	20	0/10	3.89±0.42	4.44±0.42	1.18 ± 0.76	196.7± 80.0
8.4	0.84±0.08	7.46±0.15	20	0/10	3.99±0.44	3.83±0.49	0.52 ± 0.18	150.7± 136.4
36.0	0.92±0.11	7.09±0.21	20	0/10	4.46±0.45	4.83±0.32	2.12 ± 1.03	75.2± 46.5*
121.6	0.90±0.11	6.91±0.21	20	0/10	4.96±0.29	4.30±0.38	0.10 ± 0.02*	70.9± 50.6*

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

Butylbenzyl phthalate

1.Vitellogenin Assay

Table 1. Results

Treatment (ìg/L)	Vitellogenin (ng/mg liver)		Hepatpspmatic index (%)	
	14-d	21-d	14-d	21-d
Control	0.6±0.1	1.5±0.2	2.08±0.56	1.87±0.16
14.0	0.6±0.1	1.2±0.2	2.35±0.13	1.67±0.18
26.7	0.7± 0.1	1.3±0.1	1.93±0.08	2.00±0.11
69.7	1.1± 0.2	1.5± 0.1	1.93±0.11	1.72±0.12
337.1	0.8±0.2	1.3± 0.1	2.37±0.16	2.12±0.26
1,045.4	2.6±0.5**	1.5± 0.1	2.46±0.23	2.24±0.22

Statistically significant differences from control group(**indicates p<0.01, *indicates p<0.05)

2.Partial Life---Cycle Test

Table 2-A. Results

Treatment (ìg/L)	Hatchability (%)	Time to hatching (day)	Mortality (%)	Total length (mm)	Body weight (mg)
Control	98	12.7 ± 1.0	16.3	20.1 ± 0.2	129.9 ± 3.6
0.7	94	11.1 ± 0.7	17.0	20.3 ± 0.2	137.8 ± 3.9
2.7	89	14.9 ± 1.1**	25.8	21.4 ± 0.2**	162.9 ± 4.3**
11.5	99	15.4 ± 1.1**	31.3	21.4 ± 0.2**	154.7 ± 3.8**
28.6	96	12.1 ± 0.7**	11.5	20.1 ± 0.2	131.9 ± 3.1
99.5	86	14.2 ± 1.1	30.2	22.0 ± 0.2**	179.4 ± 4.6**

Table 2-B. Results (continued)

Treatment (ìg/L)	Gonadosomatic index (%)		No. of fish	No. of males with testis—ova / No. of males	Hepatosomatic index (%)		Vitellogenin (ng/mg liver)	
	male	female			male	female	male	female
Control	0.83±0.07	7.40±0.26	20	0/10	2.14±0.15	2.52±0.19	1.12 ± 0.10	375.1± 200.6
0.7	0.96±0.11	7.60±0.21	20	0/10	2.07±0.22	2.55±0.18	1.47 ± 0.36	457.7± 164.6
2.7	1.09±0.08	7.63±0.19	20	0/10	2.68±0.29	2.99±0.24	1.43 ± 0.24	142.3± 96.7
11.5	1.12±0.08	7.43±0.28	20	0/10	2.45±0.31	3.62±0.38	1.58 ± 0.23	90.9± 28.4
28.6	1.16±0.09	7.52±0.23	20	0/10	2.81±0.37**	3.21±0.26	1.86 ± 0.40	330.4± 136.6
99.5	1.17±0.07	7.55±0.31	20	0/10	3.30±0.57	4.25±0.46	1.47 ± 0.35	129.3± 69.9

Statistically significant differences from control group(**indicates p<0.01, *indicates p<0.05)

Di-(2-ethylhexyl) adipate

1.Vitellogenin Assay

Table 1. Results

Treatment (ìg/L)	Vitellogenin (ng/mg liver)		Hepatosomatic index (%)	
	14-d	21-d	14-d	21-d
Control	0.20±0.02	0.36±0.02	2.31±0.16	2.60±0.10
2.4	0.18±0.01	0.42±0.04	2.49±0.17	2.21±0.14*
7.9	0.16± 0.05	0.38±0.03	2.77±0.21	2.30±0.12
21.5	0.18±0.01	0.37± 0.02	2.61±0.16	2.47±0.14
181.7	0.15± 0.01	0.33± 0.02	2.53±0.12	2.64±0.10
453.6	0.21±0.04	0.46±0.05	2.21±0.15	2.42±0.20

Statistically significant differences from control group(**indicates p<0.01, *indicates p<0.05)

2.Partial Life - Cycle Test

Table 2-A. Results

Treatment (ìg/L)	Hatchability (%)	Time to hatching (day)	Mortality (%)	Total length (mm)	Body weight (mg)
Control	97 ± 3.9	8.3 ± 0.1	3.5 ± 4.0	30.1 ± 2.1	260 ± 56
Solvent control	92 ± 8.4	8.4 ± 0.2	7.5 ± 8.8	31.0 ± 1.5	277 ± 51
0.711	98 ± 3.3	8.1 ± 0.2	1.8 ± 3.6	30.0 ± 2.4	261 ± 64
2.33	95 ± 3.3	8.2 ± 0.2	6.8 ± 9.4	31.0 ± 1.7	286 ± 55
7.88	92 ± 3.3	8.1 ± 0.3	13 ± 13	31.2 ± 1.9	301 ± 71**
26.3	95 ± 6.4	8.2 ± 0.1	5.1 ± 6.4	31.1 ± 1.3	280 ± 44
87.1	95 ± 6.4	8.3 ± 0.2	4.0 ± 4.6	31.1 ± 1.6	280 ± 54

Table 2-B. Results (continued)

Treatment (ìg/L)	Gonadosomatic index (%)		No. of fish	No. of males with testis-ova / No. of males	Hepatosomatic index (%)		Vitellogenin (ng/mg liver)	
	male	female			male	female	male	female
Control	1.1 ± 0.2	6.9 ± 2.9	20	0/8	1.8 ± 0.5	3.6 ± 0.604	6.3±13	2,100± 680
Solvent control	1.2 ± 0.4	9.1 ± 1.9	20	1/14	1.9 ± 0.5	3.3 ± 0.4	1.3 ± 1.6	2,500± 1,900
0.711	1.1 ± 0.4	6.7 ± 2.7	20	0/10	1.9 ± 0.4	3.1 ± 0.7	1.6 ± 2.0	2,000± 1,300
2.33	1.3 ± 0.4	8.6 ± 2.7	20	0/6	1.8 ± 0.2	3.4 ± 0.8	3.0 ± 4.7	2,600± 1,300
7.88	1.2 ± 0.2	7.5 ± 2.7	20	1/9	1.8 ± 0.3	3.7 ± 0.8	1.8 ± 1.6	1,600± 610
26.3	1.0 ± 0.3	6.7 ± 3.0	20	0/13	1.8 ± 0.5	3.6 ± 0.8	5.4 ± 9.1	2,100± 390
87.1	1.0 ± 0.2	5.7 ± 3.6	20	0/12	1.8 ± 0.3	3.1 ± 0.3	1.4 ± 1.2	1,500± 980

Statistically significant differences from control group(**indicates p<0.01, *indicates p<0.05)

Triphenyl tin chloride

1.tellogenin Assay

Table 1. Results

Treatment	Vitellogenin (ng/mg liver)		Hepatosomatic index (%)	
(ìg/L)	14-d	21-d	14-d	21-d
Control	1.6±0.3	1.7±0.2	2.61±0.23	2.71±0.49
0.118	1.1±0.1	1.2±0.2	2.72±0.17	3.55±0.38
0.280	1.4± 0.2	1.4±0.1	2.76±0.19	3.19±0.40
0.928	0.8± 0.1**	0.9± 0.1**	3.21±0.19	4.33±0.70
2.890	0.9± 0.1*	0.9± 0.1*	3.55±0.35	5.04±1.04
8.871	---	---	---	---

---indicates 100% mortality

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

2.Partial Life - Cycle Test

Table 2-A. Results

Treatment (ng/L)	Hatchability (%)	Time to hatching (day)	Mortality (%)	Total length (mm)	Body weight (mg)
Control	95	10.4 ± 0.2	9.5	21.4 ± 0.2	170.2 ± 3.9
27.6	93	10.2 ± 0.3	5.4	21.3 ± 0.2	162.6 ± 3.9
80.1	87	12.0 ± 0.4**	11.5	21.8 ± 0.1	179.6 ± 3.8
178.0	80	12.0 ± 0.5**	12.5	21.8 ± 0.2	181.5 ± 5.1
619.1	83	16.7 ± 0.9**	25.3	22.2 ± 0.2*	194.8 ± 5.9*
1,859.5	88	12.8 ± 0.6**	17.1	20.3 ± 0.2*	153.5 ± 4.7*

Table 2---B. Results (continued)

Treatment (ng/L)	Gonadosomatic index (%)		No. of fish	No. of males with testis—ova / No. of males	Hepatosomatic index (%)		Vitellogenin (ng/mg liver)	
	male	female			male	female	male	female
Control	1.14±0.08	7.57±0.17	20	0/10	3.55±0.42	4.06±0.25	1.3 ± 0.2	295.5± 117.9
27.6	1.12±0.11	7.66±0.19	20	0/10	3.50±0.40	3.96±0.30	1.6 ± 0.6	251.9± 102.1
80.1	0.98±0.09	7.54±0.22	20	0/10	3.25±0.45	3.72±0.36	1.1 ± 0.1	276.0± 99.5
178.0	1.08±0.07	7.53±0.22	20	0/10	2.65±0.18	3.93±0.29	1.2 ± 0.1	335.0± 150.7
619.1	1.05±0.09	7.22±0.20	20	0/10	3.46±0.39	5.12±0.23	1.4 ± 0.5	183.6± 77.7
1,859.5	1.07±0.11	7.29±0.18	20	0/10	4.38±0.30	4.91±0.41	1.2 ± 0.4	43.2± 20.9

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

Benzophenone

1.Vitellogenin Assay

Table 1. Results

Treatment (ìg/L)	Vitellogenin (ng/mg liver)		Hepatosomatic index (%)	
	14-d	21-d	14-d	21-d
Control	ND	ND	1.63±0.38	1.71±0.34
Solvent control	ND	ND	1.54±0.30	1.82±0.41
48	ND	ND	1.67±0.38	1.80±0.41
160	ND	ND	1.62±0.25	2.03±0.34
500	4.7±5.9**	2.3±3.0**	1.66±0.36	2.02±0.50
1,380	700±480**	1,600±950**	2.04±0.43**	2.21±0.56
4,650	4,600±2,900**	5,400±2,600**	2.13±0.57**	2.27±0.92*

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

2.Partial Life---Cycle Test

Table 2-A. Results

Treatment (ìg/L)	Hatchability (%)	Time to hatching (day)	Mortality (%)	Total length (mm)	Body weight (mg)
Control	100 ± 0	9.4 ± 0.4	10 ± 3.9	29.5 ± 1.9	254 ± 49
5.06	100 ± 0	9.2 ± 0.1	8.3 ± 6.4	29.5 ± 1.6	253 ± 45
15.1	97 ± 3.9	9.3 ± 0.2	3.5 ± 4.0	29.5 ± 1.6	252 ± 39
47.0	93 ± 7.7	9.3 ± 0.1	8.9 ± 7.0	30.0 ± 1.4	270 ± 40
144	98 ± 3.3	9.3 ± 0.3	3.3 ± 3.9	29.6 ± 1.3	264 ± 33
435	98 ± 3.3	9.5 ± 0.3	1.7 ± 3.3	30.1 ± 1.6	265 ± 42

Table 2---B. Results (continued)

Treatment (ìg/L)	Gonadosomatic index (%)		No. of fish	No. of males with testis—ova / No. of males	Hepatosomatic index (%)		Vitellogenin (ng/mg liver)	
	male	female			male	female	male	female
Control	0.97 ± 0.3	4.0 ± 3.1	20	0/7	2.3 ± 0.6	4.1 ± 0.9	10 ± 9.3	1,800± 1,600
5.06	0.55 ± 0.2	4.5 ± 2.9	20	1/10	2.3 ± 0.5	4.5 ± 0.7	17 ± 17	2,300± 1,600
15.1	0.77 ± 0.2	3.0 ± 2.6	20	0/8	2.6 ± 0.6	4.1 ± 0.7	5.5 ± 9.3	2,100± 1,400
47.0	0.64 ± 0.4	6.0 ± 2.5	20	2/11	3.0 ± 0.7*	3.7 ± 0.6	6.4 ± 4.9	3,100± 1,600
144	0.58 ± 0.2*	3.6 ± 3.4	20	0/11	2.2 ± 0.4	3.3 ± 0.9	3.3 ± 3.2	2,200± 1,400
435	0.88 ± 0.4	6.2 ± 2.8	20	1/11	2.3 ± 0.4	3.8 ± 0.4	56 ± 69	3,700± 2,000

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

Octachlorostyrene

1.Vitellogenin Assay

Table 1. Results

Treatment (ìg/L)	Vitellogenin (ng/mg liver)		Hepatosomatic index (%)	
	14-d	21-d	14-d	21-d
Control	ND	ND	1.33±0.29	1.61±0.27
Solvent control	ND	ND	1.38±0.30	1.51±0.20
0.24	ND	ND	1.40±0.38	1.42±0.28
0.49	ND	ND	1.56±0.21	1.67±0.41
1.1	ND	ND	1.39±0.30	1.56±0.21
2.8	ND	ND	1.36±0.32	1.53±0.27
6.6	ND	ND	1.53±0.27	1.46±0.15

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)

2.Partial Life Cycle Test

Table 2---A. Results

Treatment (ìg/L)	Hatchability (%)	Time to hatching (day)	Mortality (%)	Total length (mm)	Body weight (mg)
Control	97 ± 3.9	9.1 ± 0.2	1.8 ± 3.6	30.5 ± 1.8	267 ± 61
Solvent control	97 ± 3.9	9.2 ± 0.1	6.8 ± 5.5	30.8 ± 1.9	279 ± 55
0.0519	95 ± 6.4	9.1 ± 0.1	1.8 ± 3.6	29.9 ± 1.7	280 ± 44
0.148	98 ± 3.3	9.0 ± 0.1	7.1 ± 10	30.4 ± 1.6	274 ± 48
0.388	95 ± 3.3	9.1 ± 0.2	0 ± 0	30.5 ± 2.6	282 ± 60
1.30	95 ± 3.3	9.1 ± 0.1	0 ± 0	30.5 ± 1.8	269 ± 53
5.31	98 ± 3.6	9.0 ± 0.0	12 ± 9.2	30.2 ± 1.4	259 ± 45

Table 2---B. Results (continued)

Treatment (ìg/L)	Gonadosomatic index (%)		No. of fish	No. of males with testis-ova / No. of males	Hepatosomatic index (%)		Vitellogenin (ng/mg liver)	
	male	female			male	female	male	female
Control	0.82 ± 0.3	4.7 ± 3.5	20	0/11	2.8 ± 0.3	3.5 ± 0.9	6.6 ±11.1	1,100± 980
Solvent control	0.78 ± 0.2	3.7 ± 3.8	20	0/9	2.8 ± 0.5	3.9 ± 1.0	7.7 ± 8.8	980± 1,100
0.0519	0.82 ± 0.3	3.5 ± 3.0	20	0/7	2.2 ± 0.8	3.7± 0.9	2.0 ± 2.0	1,600± 1,400
0.148	0.84 ± 0.6	4.9 ± 4.0	20	0/13	2.0 ± 0.8**	3.6 ± 0.8	1.5 ± 3.3**	1,600± 1,300
0.388	0.84 ± 0.4	5.2 ± 4.0	20	0/12	2.5 ± 0.6	3.9 ± 0.8	1.2 ± 1.8*	1,800± 1,200
1.30	0.82 ± 0.2	3.9 ± 3.6	20	0/9	2.5 ± 0.7	3.9 ± 1.1	5.0 ± 6.1	1,500± 1,100
5.31	0.70 ± 0.3	7.7 ± 3.5	20	0/13	2.6 ± 0.7	4.3 ± 1.0	0.3 ± 0.3**	1,500± 660

Statistically significant differences from control group(**indicates $p<0.01$, *indicates $p<0.05$)