

LIFE-CYCLE TOXICITY OF 4-NONYLPHENOL TO MEDAKA (*ORYZIAS LATIPES*)HIROFUMI YOKOTA,\*† MASANORI SEKI,† MASANOBU MAEDA,† YUJI OSHIMA,‡ HIROSHI TADOKORO,†  
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**Abstract**—We studied the chronic effects of 4-nonylphenol (4-NP) on reproductive status of medaka (*Oryzias latipes*) over two generations of continuous exposure. The exposure study of the parental ( $F_0$ ) medaka was begun on embryos within 24 h postfertilization and continued with monitoring through embryological development, hatching, posthatch survival, growth, sexual differentiation, and reproduction under flow-through exposures to mean measured 4-NP concentrations of 4.2, 8.2, 17.7, 51.5, and 183  $\mu\text{g/L}$  for up to 104 d. Eggs spawned from the  $F_0$  fish at 102 and 103 d posthatch were also examined for hatchability, survival after hatching, growth, and sexual differentiation until 60 d posthatch. The 183- $\mu\text{g/L}$  treatment significantly reduced the embryo survival and swim-up success of the  $F_0$  fish. The cumulative mortality after swim-up of the  $F_0$  fish exposed to 17.7 and 51.5  $\mu\text{g/L}$  were significantly higher than the control mortality. No concentration-related effect of 4-NP was observed on the growth of surviving  $F_0$  fish at 60 d posthatch. However, the sex ratio estimated from the appearance of their secondary sex characteristics was skewed toward female in the 51.5- $\mu\text{g/L}$  treatment. Additionally, gonadal histology showed that 20% of the fish in the 17.7- $\mu\text{g/L}$  treatment and 40% in the 51.5- $\mu\text{g/L}$  treatment had testis-ova, indicating that 4-NP affects the gonadal development and survival of medaka at similar concentrations in juveniles. The sex ratio of the  $F_0$  fish in the 51.5- $\mu\text{g/L}$  treatment was completely skewed toward female; subsequently, the effects on fecundity and fertility in this generation were monitored at mean measured concentrations of 4.2, 8.2, and 17.7  $\mu\text{g/L}$  from 71 to 103 d posthatch. Fecundity was unaffected by any of the treatments examined. The mean fertility in the 17.7- $\mu\text{g/L}$  treatment was reduced to 76% of that in the controls, although no statistically significant differences were determined. Overall, these results indicate that the lowest-observed-effect concentration (LOEC) and no-observed-effect concentration (NOEC) of 4-NP through the life cycle of the  $F_0$  medaka were 17.7 and 8.2  $\mu\text{g/L}$ , respectively. In the  $F_1$  medaka, no significant effects were observed on hatching success, posthatch mortality, or growth, but sexual differentiation at 60 d posthatch was affected. Induction of testis-ova in the gonads of the  $F_1$  fish was observed in both the 8.2- and the 17.7- $\mu\text{g/L}$  concentrations. The results indicate that 4-NP can have significant effects on reproductive potential of medaka at concentrations as low as 17.7  $\mu\text{g/L}$ .

**Keywords**—4-Nonylphenol    Environmental estrogen    Medaka    Testis-ova    Life-cycle toxicity

## INTRODUCTION

Global concern about environmental pollution of exogenous estrogenic substances has increased over the past two decades. These substances have the potential to interfere with the endocrine system of wildlife, resulting in altered sexual development and reproduction [1]. Endocrine-disrupting effects have been well reported in aquatic fish species in association with the discharge of treated sewage to rivers in several countries [2–6]. Throughout the United Kingdom, large populations of roach (*Rutilus rutilus*) inhabiting the settlement lagoons of sewage treatment works (STWs) have hermaphroditic gonads composed of both testicular germ cells and oocytes [7]. Domestic semi-field studies have confirmed that most effluents from STWs can exert estrogenic activity by the induction of vitellogenin, a female-specific protein, in caged male rainbow trout (*Oncorhynchus mykiss*) [8]. Although a study using bioassay fractionation techniques has identified that natural and synthetic hormones (17 $\beta$ -estradiol, estrone, and ethynylestradiol) are responsible for the estrogenicity of effluents from seven STWs in the United Kingdom [9], the participation of alkylphenolic chemicals has been also implicated [2,8].

Alkylphenol polyethoxylates are widely used as nonionic surfactants in the manufacturing of cleaning agents, plastics, paper, cosmetics, and food products [10]. Nonylphenol ethoxylates (NPEOs) have been used predominantly, amounting to about 80% of the production of alkylphenol surfactants. Although the NPEOs in drainage flowing into STWs are nontoxic and hydrophilic, bacterial degradation produces toxic hydrophobic compounds such as nonylphenols (NPs) during sewage treatment [11]. The detected concentrations of NPs are generally very low (<1  $\mu\text{g/L}$ ), but in some sites they exceed 10  $\mu\text{g/L}$  [12–17].

In both in vitro and in vivo assays, alkylphenolic chemicals, including NPs, have been shown to possess estrogenic activity [18–21]. Several studies using intact fish have confirmed that NPs have physiological and developmental effects related to their estrogenic properties. The exposure of sexually mature male rainbow trout to alkylphenol polyethoxylates, including 4-nonylphenol (4-NP), caused a dose-dependent elevation of plasma vitellogenin levels accompanied by a reduction in testicular weight [21]. Christiansen et al. [22] found that treatment with 4-*tert*-NP caused a significant increase in vitellogenin synthesis and a marked reduction in the gonadosomatic index (GSI) of male eelpout (*Zoarces viviparus*). Gray and Metcalfe [23] reported that treatment with *p*-NP induced testis-ova in Japanese medaka (*Oryzias latipes*). Many studies have been

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conducted on the ecotoxicity of NPs, and these data have been reviewed by Staples et al. [24]. These authors pointed out that the threshold NP levels required to produce adverse effects in fish exceed the concentrations observed in rivers and lakes and even in most sewage effluents. However, no reports have been made of how continuous exposure to NPs may affect the entire life cycle, including the sexual differentiation and reproductive phases. Further work needs to be done to elucidate dose–response relationships and to determine NOECs for the chronic effects of NPs on the full life cycle of fish, including the developmental and reproductive phases.

Medaka is an ideal test species for evaluating life-cycle toxicity in the parental and progeny generations because of its short life cycle [25]. This fish can spawn 10 to 40 eggs daily under optimal conditions, which facilitates studies of reproductive effects. Medaka is also sensitive to estrogenic substances because it typically develops an intersex condition (testis–ova) in the gonadal tissue when exposed to estrogenic chemicals [23,26,27]. Multigeneration toxicity testing in medaka can be valuable and definitive for determining the estrogenic effects of synthetic chemicals and substances present in the aquatic environment on the complete life cycles of fish.

This study was conducted to elucidate the chronic effects of 4-NP on the life cycle of medaka over two generations in continuous exposures. We examined embryological development, hatching success, posthatch survival, growth, sexual differentiation, and reproduction of the  $F_0$  generation as well as those of the progeny generation ( $F_1$ ).

## MATERIALS AND METHODS

### 4-NP

The test substance (analytical grade 4-NP; 97.4% purity as a mixture of isomers) was obtained from Kanto Chemical, Tokyo, Japan. A 4-NP stock solution of 1,500 mg/L was prepared by dissolving in ethanol.

### Test fish

Medaka were originally purchased from a local fish farm in Kumamoto, Japan, and a breeding stock of these medaka has been maintained for several years in our laboratory. About 100 breeding medaka pairs (mean  $\pm$  standard deviation of body wt and total length were  $233 \pm 50$  mg and  $29.9 \pm 1.8$  mm, respectively) were kept for at least two weeks in 1-L chambers with flow-through dechlorinated tap water (pH, 7.4–7.5; hardness, 44.0–73.5 mg  $\text{CaCO}_3/\text{L}$ ) at  $24 \pm 1^\circ\text{C}$ . During mating, the fish were placed under a summer photoperiod (16:8-h light:dark) and fed exclusively with *Artemia* nauplii (<24 h after hatching) twice a day. Eggs spawned from each female were carefully collected within a few hours after fertilization, pooled in a petri dish containing about 5 ml of dechlorinated tap water, checked for fertilization and development (to the early morula stage) under a light microscope, and then subjected to the 4-NP exposure.

### Exposure design

The exposure system consisted of a continuous-flow mini-diluter system modified from Benoit et al. [28]. The 4-NP stock solution was injected in this exposure system by a mini-chemical pump (SP-D-2000; Nihon Seimitsu Kagaku, Tokyo, Japan). The flow rate of the stock solution was calibrated in accordance with our analytical confirmation of the test solutions before the initiation of exposure. The stock solution and dechlorinated tap water were delivered to a mixing vessel be-

fore entering a cylindrical glass test chamber (diameter, 15.0 cm; depth, 17.5 cm). The test chamber was designed to contain about 1.8 L of the test solution by maintaining an overflow level of 10 cm. The flow rate of dechlorinated tap water was checked volumetrically once a day. The renewal rate of the test solution in each chamber was 14 times a day, and the stock solution was newly prepared daily. Nominal treatment concentrations of 4-NP at 1.85, 5.56, 16.7, 50, and 150  $\mu\text{g}/\text{L}$  were selected for this study based on a 7-d preliminary exposure with newly hatched larvae. The controls received tap water alone, and the solvent controls received dechlorinated tap water containing ethanol (100  $\mu\text{L}/\text{L}$ ) at a concentration equivalent to that of the test solution in the highest 4-NP treatment. The photoperiod was 16 h light: 8 h dark. Water temperature was controlled with a thermostat and kept at  $24 \pm 1^\circ\text{C}$  for the reproductive phase, when temperatures were increased to  $28 \pm 1^\circ\text{C}$ . The test equipment and chambers were cleaned at least twice a week to prevent dense bacterial growth. Residual food and feces in the test chamber were removed daily.

### Biological protocols

**Embryological phase.** Exposure was initiated at less than 24 h postfertilization. The 60 embryos employed for each treatment were randomly separated into four groups of 15 in each test chamber for testing in quadruplicate. The developing embryos were observed daily under a stereoscopic microscope. Any dead embryos were discarded; live ones were returned to the chamber. This procedure was repeated until all the living embryos had hatched.

**Larval–juvenile phase.** After hatching, the larvae were fed an adequate amount of *Artemia* nauplii (<24 h after hatching) twice a day; some nauplii remained for at least 1 h after feeding. Daily observation was made to examine mortality and abnormal behavior and appearance until 60 d posthatch, and dead fish were removed as soon as possible.

At 60 d posthatch, five individuals from each of the four test chambers (i.e., 20 individuals from each treatment group, except for the highest treatment group in which complete mortality occurred), were randomly removed and observed for external secondary sex characteristics based on the shape of the dorsal and anal fins. The fish were euthanized with an overdose solution (a 1,000-fold aqueous solution) of anesthetic FA-100 (Tanabe Seiyaku, Osaka, Japan) and drained on filter paper; body weight and total length were measured. They were then fixed in Bouin's solution and prepared for gonadal histology according to standard procedures [29]. The fish embedded in paraffin wax were sectioned longitudinally at 3  $\mu\text{m}$  in thickness providing at least five sections per gonad. The sections were stained with hematoxylin and eosin, mounted with malinol (Muto pure chemicals, Tokyo, Japan), and then examined under a light microscope.

**Reproductive phase.** At 70 d posthatch, the sex of the surviving fish was distinguished by their external appearances, and six mating pairs from each of the two low treatments (4.2 and 8.2  $\mu\text{g}/\text{L}$ ) and the controls and solvent controls were selected to examine fecundity and fertility. No pairs from the 51.5- $\mu\text{g}/\text{L}$  treatment and only three pairs from the 17.7- $\mu\text{g}/\text{L}$  treatment could be selected because of a skewed sex ratio and/or the limited number of surviving fish. Each pair was assigned to a test chamber and exposed until 104 d posthatch. The water temperature was increased from  $24^\circ\text{C}$  to  $28^\circ\text{C}$  to stimulate spawning. The eggs spawned from each female were collected, counted, and assessed for viability microscopically for 33 con-

secutive days (71–103 d posthatch). On the day after the end of the reproductive examination (104 d posthatch), all pairs examined were removed from their chambers, and their body weights and total lengths were measured after overdosing with FA-100. Their gonads were removed and weighed, and the GSI was calculated (i.e.,  $100 \times \text{gonad weight/body weight}$ ).

***F<sub>1</sub> embryo phase.*** The eggs spawned on the last 2 d of the reproductive phase (102 and 103 d posthatch) were subjected to the following semistatic 4-NP exposure until hatching. The fertilized eggs from each spawning in the 4.2-, 8.2-, and 17.7- $\mu\text{g/L}$  treatments and the controls and solvent controls were collected and placed in a 100-ml beaker containing 80 ml of test solution and incubated in a water bath at  $24 \pm 1^\circ\text{C}$ . The test solutions were prepared by dilution of 4-NP stock solution with dechlorinated tap water and renewed every day until the last hatching. In the control and the solvent control treatments, tap water alone and tap water containing ethanol (100  $\mu\text{l/L}$ ) were used. The developing embryos were observed daily and dead ones removed.

***F<sub>1</sub> larvae-juvenile phase.*** The newly hatched larvae in the two low treatments (4.2 and 8.2  $\mu\text{g/L}$ ) and the controls and solvent controls were randomly transferred to four test chambers in each treatment and kept in the chambers until the last hatching. Then 15 larvae in each chamber were selected (60 larvae in each treatment group). In the 17.7- $\mu\text{g/L}$  treatment, the larvae were transferred to two test chambers because of fewer embryos from three pairs of  $F_0$  fish, and 15 larvae in each chamber were selected (30 larvae in this treatment group). Then they were treated until 60 d posthatch in the same flow-through system described previously for the  $F_0$  generation. The fish were checked daily until 60 d posthatch for mortality, abnormal behavior, and appearance. Dead fish were removed as soon as possible.

At 60 d posthatch, the external secondary sex characteristics of all the surviving  $F_1$  fish were observed, and their weights and lengths were measured after overdosing with FA-100. They were then fixed in Bouin's solution and prepared for gonadal histology as previously described for the  $F_0$  generation.

#### Determination of 4-NP concentration in test solution

**Sample preparation.** The concentration of 4-NP in each test solution was determined approximately every two weeks through the exposure period, except for the semistatic period of  $F_1$  embryo exposure. Equal volumes of test solution collected from all test chambers of each treatment were combined and applied individually to preconditioned Sep-Pak PS-2 Plus solid-phase extraction cartridges (Waters, Tokyo, Japan). The 4-NP was eluted with 6 ml of methyl acetate. The eluate was centrifuged (3,000 rpm, 10 min) and the methyl acetate phase collected. Next, 5 ml of hexane were added to the water phase, and the mixture was shaken for 1 min. The hexane phase was combined with the collected methyl acetate phase, and then the solvents were removed by drying in a stream of nitrogen. The residue was dissolved in 1 ml of the hexane solution containing 50  $\mu\text{g/ml}$  of 4-*tert*-octylphenol as an internal standard and then analyzed by gas chromatography/mass spectrometry (GC-MS). The calibration curves were plotted as the ratios of the peak areas of 4-NP to that of the internal standard.

**GC-MS conditions.** The samples were analyzed by GC-MS with a Shimadzu QP-5,000 equipped with a 30-m DB-1 column (250- $\mu\text{m}$  i.d., film thickness 0.25  $\mu\text{m}$ ; J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a flow rate

Table 1. Geometric mean concentrations of 4-nonylphenol measured in the test solutions through the exposure period

Nominal concn. ( $\mu\text{g/L}$ )	<i>N</i>	Mean measured concn. ( $\mu\text{g/L}$ )	SD <sup>a</sup>	CV <sup>b</sup> (%)
Control	12	ND <sup>c</sup>		
Solvent control	12	ND		
1.85	12	4.2	1.6	38.5
5.56	12	8.2	3.7	44.6
16.7	12	17.7	3.4	19.1
50	6	51.5	7.1	13.8
150	2	183		

<sup>a</sup> SD = standard deviation.

<sup>b</sup> CV = coefficients of variation.

<sup>c</sup> ND = not determined at 0.5  $\mu\text{g/L}$ .

of 50 ml/min. One-microliter injections were made in a splitless mode with a 1-min purge-off. The injection-port temperature was maintained at  $280^\circ\text{C}$  to ensure complete volatilization of the sample. The temperature program for the column began with a 1-min hold at  $80^\circ\text{C}$ , followed by a  $30^\circ\text{C/min}$  ramp-up to  $180^\circ\text{C}$ , a  $5^\circ\text{C/min}$  ramp-up to  $200^\circ\text{C}$ , and a  $35^\circ\text{C/min}$  ramp-up to  $280^\circ\text{C}$ , which was held for 5 min. After being eluted from the GC column, the samples were carried through a  $250^\circ\text{C}$  transfer line into the ion source of the mass spectrometer held at  $250^\circ\text{C}$ . The electron energy was 70 eV and electron multiplier voltage 1,000 V. Ions of 4-NP were monitored at  $m/z$  107 and 135 and the internal standard at  $m/z$  135.

#### Statistical analysis

Either a chi-square test, Student's *t* test (parametric data), or Bonferroni's *U* test (nonparametric data) were used prior to data analysis to determine whether differences existed between the solvent control and control groups. If no differences were found, these groups were pooled for subsequent analysis. If differences were found, the control group without solvent was excluded from the subsequent analyses. The experimental data, except for the sex ratios in each treatment for the  $F_0$  and  $F_1$  generations, were checked for homogeneity of variances and assumptions of normality across treatments by Levene's test and Shapiro-Wilk's test, respectively [30,31]. When the assumptions were met, the data were subjected to analysis of variance followed by Dunnett's multiple comparison test [32]. When the data failed to meet either one or both of the assumptions, a nonparametric Wilcoxon's rank sum test was used with Bonferroni's adjustment [33]. The data on hatchability, swim-up failure, and cumulative mortality were transformed to arcsine for variance stabilization before analysis [34]. The data on sex ratios distinguished from the gonads were assessed by chi-square analysis. All statistical analyses were conducted with SPSS® Base 8.0 J [30]. Differences were considered to be significant at the  $p \leq 0.05$  level.

## RESULTS

#### Concentration of 4-NP in the test solution

The 4-NP nominal concentrations  $>16.7 \mu\text{g/L}$  remained consistent throughout the exposure period (Table 1). However, the two low nominal concentrations (1.85 and 5.56  $\mu\text{g/L}$ ) were not stable, and the geometric mean concentrations in these treatments were 4.2  $\mu\text{g/L}$  ( $CV = 38.5\%$ ) and 8.2  $\mu\text{g/L}$  ( $CV = 44.6\%$ ) (Table 1). The concentrations in the control and solvent control treatments were all below the determination limit ( $<0.5 \mu\text{g/L}$ ).

Table 2. Mean  $\pm$  standard deviation ( $n = 4$ ) of hatchability, time to hatch, and swim-up failure in the  $F_0$  generation

4-Nonylphenol concn. ( $\mu\text{g/L}$ ) <sup>a</sup>	Hatchability (%)	Time to hatch (d)	Swim-up failure (%)
Control	90.0 $\pm$ 8.6	9.4 $\pm$ 0.2	5.5 $\pm$ 6.9
Solvent control	93.3 $\pm$ 5.4	9.9 $\pm$ 0.3	7.0 $\pm$ 5.8
4.2	95.0 $\pm$ 6.4	9.5 $\pm$ 0.02	3.6 $\pm$ 4.2
8.2	90.0 $\pm$ 8.6	9.6 $\pm$ 0.2	16.2 $\pm$ 7.7
17.7	88.3 $\pm$ 6.4	10.2 $\pm$ 0.4	18.7 $\pm$ 9.7
51.5	93.3 $\pm$ 7.7	10.2 $\pm$ 0.6	14.1 $\pm$ 5.1
183	46.7 $\pm$ 18.1 <sup>b</sup>	9.6 $\pm$ 0.3	100 <sup>c</sup>

<sup>a</sup> Mean measured concentrations.

<sup>b</sup> Significantly different from the pooled controls ( $p < 0.001$ ).

<sup>c</sup> Significantly different from the pooled controls ( $p = 0.004$ ).

### $F_0$ generation

**Mortality, abnormal behavior, and appearance.** Embryo development and hatching of medaka eggs were affected by 4-NP exposure. Hatchability was significantly decreased in the highest treatment (183  $\mu\text{g/L}$ ) relative to the pooled controls ( $p < 0.001$ ), but most hatchabilities in the lower treatments were  $>90\%$ , with no significant difference from the controls (Table 2). In the 183- $\mu\text{g/L}$  treatment, about 50% of embryos died after 8 d postfertilization, exhibiting a reduction in their heart rate and blood circulation before dying. The time to hatching was about 10 d in all treatments (Table 2). However, no hatched larvae swam up successfully in the 183- $\mu\text{g/L}$  treatment, resulting in a significant difference from the swim-up data for the pooled controls ( $p = 0.004$ ) (Table 2).

Post-swim-up mortality was positively correlated with 4-NP concentration (mortality =  $12.1 \log e [4\text{-NP } \mu\text{g/L}] - 14.7$ ,  $r^2 = 0.99$ ). Mortalities in the 17.7- and 51.5- $\mu\text{g/L}$  treatments increased after 20 d posthatch, and the cumulative mortalities at 60 d posthatch were significantly different from those of the pooled controls ( $p = 0.031$  and  $0.002$ , respectively) (Fig. 1). Dying fish displayed a lightened body color. Although a few dead fish were also observed in the two low treatments and the controls during exposure, their cumulative mortalities were  $<10\%$  at 60 d posthatch, and no differences were observed between these treatments and the pooled controls.

**Growth, external secondary sex characteristics, and gonadal histology.** No significant differences were observed in either mean total length or body weight of the  $F_0$  fish at 60 d posthatch in any treatments (Table 3).

From our observation of external secondary sex character-

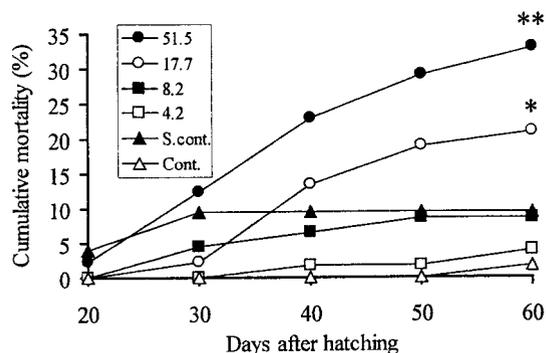


Fig. 1. Post-swim-up cumulative mortality from 20 to 60 d posthatch in each 4-nonylphenol (4-NP) treatment (4.2–51.5  $\mu\text{g/L}$ ) of the  $F_0$  generation. Concentrations are expressed as mean measured concentrations ( $\mu\text{g/L}$ ). \* and \*\* denote significant differences from the pooled controls at  $p = 0.031$  and  $0.002$ , respectively.

istics, the sex ratios of males to females in the controls were about 1:1.3, and those in the three groups treated with 4-NP at  $\leq 17.7 \mu\text{g/L}$  were slightly different from each other, whereas no males were distinguished in the 51.5- $\mu\text{g/L}$  treatment (Table 3). This result was not statistically analyzed because external secondary sex characteristics may not be reliable for intersex medaka.

The induction of testis-ova composed of both testicular germ cells and oocytes in the gonad was observed in the 17.7- and 51.5- $\mu\text{g/L}$  treatments but not at lower concentrations. The sex ratios determined by histological examination were similar to those found on external examination (Table 3). In the 17.7- $\mu\text{g/L}$  treatment, 4 of 20 fish (20%) had testis-ova, and all these fish displayed externally male characteristics. Moreover, 8 of 20 fish (40%) in the 51.5- $\mu\text{g/L}$  treatment had progressed testis-ova and exhibited externally female characteristics (Table 3). The extent of oocyte-containing parenchyma in each testis-ova specimen increased with increasing of 4-NP concentrations (Fig. 2). Although the oocytes in testis-ova observed in the 17.7- $\mu\text{g/L}$  treatment occurred frequently and were often in clusters, spermatocytes and spermatids could still be differentiated, and spermatogenesis was observed (Fig. 2A). In testis-ova of the 51.5- $\mu\text{g/L}$  treatment, almost the whole area was composed of oocytes that accompanied small testicular tissues interspersed with few testicular germ cells (Fig. 2B). Furthermore, abnormal connective tissues were observed in the testis-ova gonads. However, no histological abnormalities were observed in the ovaries of medaka in the 17.7- and 51.5- $\mu\text{g/L}$

Table 3. Total length and body weight of  $F_0$  fish at 60 d posthatch and their sex ratios as determined by gross examination of secondary sex characteristics and by gonadal histology

4-Nonylphenol concn. ( $\mu\text{g/L}$ ) <sup>a</sup>	Total length (mm) <sup>b</sup>	Body weight (mg) <sup>b</sup>	Sex ratio ( $\delta:\eta$ )	Gonadal histology		
				Number of fish with		
				Testis	Ovary	Testis-ova
Control	26.4 $\pm$ 1.7	178 $\pm$ 36	9:11	9	11	0
Solvent control	25.9 $\pm$ 1.8	170 $\pm$ 45	8:12	8	12	0
4.2	26.0 $\pm$ 1.4	167 $\pm$ 33	12:8	12	8	0
8.2	26.9 $\pm$ 2.0	197 $\pm$ 44	13:7	14	6	0
17.7	26.1 $\pm$ 2.3	179 $\pm$ 48	9:11	5	11	4
51.5	25.2 $\pm$ 1.9	167 $\pm$ 42	0:20	0 <sup>c</sup>	12 <sup>c</sup>	8 <sup>c</sup>

<sup>a</sup> Mean measured concentrations.

<sup>b</sup> Data expressed as mean  $\pm$  standard deviation ( $n = 20$ ).

<sup>c</sup> The sex ratio obtained from gonadal histology differed significantly from that of the pooled controls ( $p < 0.001$ ).

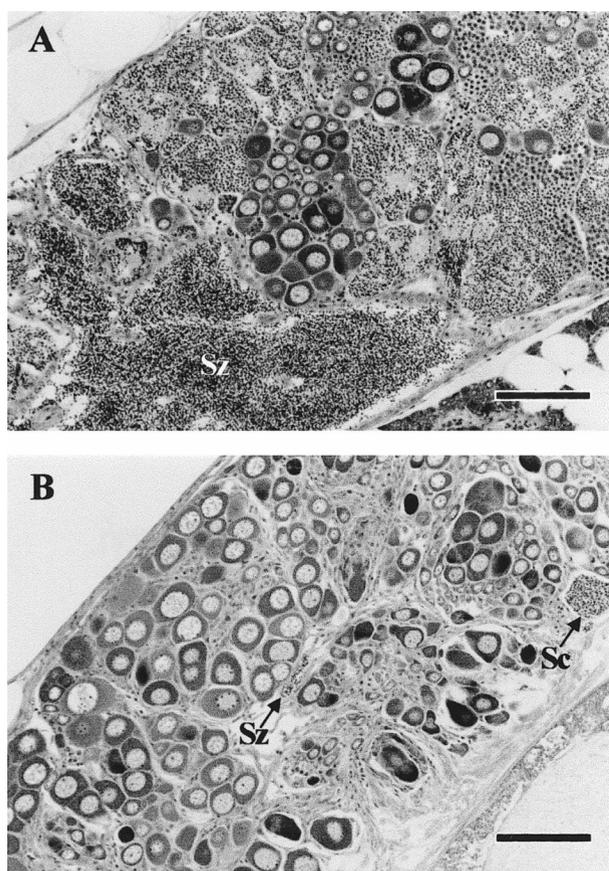


Fig. 2. Longitudinal sections of testis-ova in the gonads of  $F_0$  generation medaka in the 17.7- $\mu\text{g/L}$  (A) and 51.5- $\mu\text{g/L}$  (B) treatment at 60 d posthatch. Each bar shows 100  $\mu\text{m}$  length. (A) Oocytes appear in clusters within the testicular tissue. Numerous spermatozoa (Sz) are still present in a compacted mass in this section. (B) A more progressed testis-ova. Almost the entire area is composed of oocytes, accompanying small testicular tissues interspersed with a few spermatozoa (Sz) and spermocytes (Sc).

treatments. The gonads of medaka treated with  $<17.7 \mu\text{g/L}$  appeared histologically identical to those of the controls. Chi-square analysis indicated a significant difference between the sex ratios obtained from histological examination of the pooled controls and of the fish in the 51.5- $\mu\text{g/L}$  treatment ( $p < 0.001$ ).

**Reproduction.** The fecundity of paired medaka during the reproductive phase from 71 to 103 d posthatch was not affected by 4-NP treatment; however, mean fertility was decreased in the 17.7- $\mu\text{g/L}$  treatment (Fig. 3). The breeding pairs in the two low treatments and in the controls spawned every day, and their mean fertility maintained  $>90\%$ . In the 17.7- $\mu\text{g/L}$  treatment, however, the fertility in one of three pairs declined from 14 d after initiation of the reproductive phase (94 d posthatch), and thereafter few fertilized eggs were observed in this mating pair. Consequently, the mean fertility of the breeding pairs in the 17.7- $\mu\text{g/L}$  treatment was reduced to 76% of the control pairs, but no statistically significant difference was determined because of the small sample size ( $n = 3$ ).

The GSI of male medaka at the end of the reproductive phase was reduced in the 17.7- $\mu\text{g/L}$  treatment, although no statistically significant difference was found because the values within this treatment varied considerably (Fig. 4A). The GSI in female fish increased with an increase in 4-NP concentration, resulting in a significant elevation of GSI in the fish

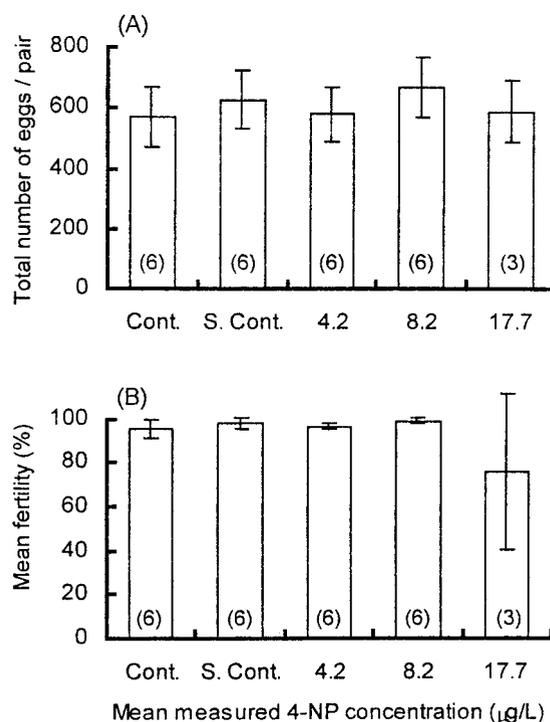


Fig. 3. Fecundity (A) and fertility (B) of paired  $F_0$  generation medaka in the reproductive phase from 71 to 103 d posthatch. Data were expressed as mean  $\pm$  standard deviation. The number of pairs in each treatment was indicated on each bar.

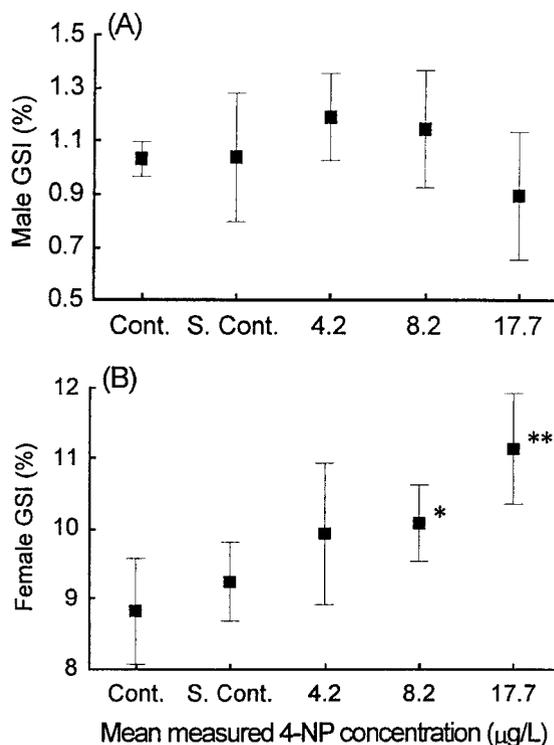


Fig. 4. Gonadosomatic index (GSI) in male (A) and female (B) of paired medaka ( $F_0$ ) at the end of the reproductive phase. Data were expressed as mean  $\pm$  standard deviation. The sample size in each treatment was 6, except in the 17.7- $\mu\text{g/L}$  treatment ( $n = 3$ ). \* and \*\* denote significant differences from the pooled controls at  $p = 0.050$  and 0.002, respectively.

Table 4. Hatchability and time to hatch in F<sub>1</sub> embryos collected during the last 2 d of the reproductive phase of F<sub>0</sub><sup>a</sup>

4-Nonylphenol concn. (μg/L) <sup>b</sup>	Hatchability (%) of F <sub>1</sub> embryo (days to hatch)				
	<i>n</i> <sup>c</sup>	From F <sub>0</sub> at 102 d posthatch	<i>N</i> <sup>c</sup>	From F <sub>0</sub> at 103 d posthatch	Mean
Control	99	64.8 ± 33.9 (10.5 ± 0.4)	114	84.9 ± 18.8 (10.0 ± 0.4)	74.4 (10.2)
Solvent control	139	76.3 ± 35.3 (11.0 ± 0.6)	115	76.5 ± 15.7 (10.4 ± 0.3)	76.4 (10.7)
4.2	125	80.5 ± 13.0 (10.7 ± 0.5)	95	67.5 ± 22.5 (10.1 ± 0.6)	74.0 (10.4)
8.2	101	71.8 ± 21.0 (11.2 ± 0.8)	130	81.9 ± 16.2 (10.0 ± 0.5)	76.9 (10.6)
17.7	35	73.4 ± 9.4 (12.2 ± 0.2)	35	97.5 ± 3.5 (11.1 ± 0.4)	85.4 (11.6)

<sup>a</sup> Data expressed as mean ± standard deviation (*n* = 3 in the 17.7-μg/L treatment and *n* = 6 in the others).

<sup>b</sup> Mean measured concentrations.

<sup>c</sup> Number of fertilized eggs.

treated with 4-NP at 8.2 and 17.7 μg/L (compared with that in the pooled controls) (Fig. 4B).

### F<sub>1</sub> generation

**Mortality, abnormal behavior, and appearance.** No embryological abnormalities or hatching failures of fertilized eggs (F<sub>1</sub> embryos) were observed in any treatment (Table 4). The hatchabilities in all treatments were >70%, and the time to hatch in most embryos was 10 to 12 d in every treatment, with no significant differences between the treatments and the pooled controls (Table 4).

Although a few dead fish were observed in all treatments, the F<sub>1</sub> juvenile mortality rate for 60 d posthatch was not significantly higher than that of the pooled controls (Table 5).

**Growth, external secondary sex characteristics, and gonadal histology.** The growth of 4-NP-exposed F<sub>1</sub> juveniles at 60 d posthatch was not affected at the concentrations tested. The mean total lengths (range 25.9–27.0 mm) and body weights (range 169–178 mg) of fish exhibited no significant differences between treatments and controls (Table 5).

Morphological examination of secondary sex characteristics showed that the sex ratios were approximately 1:1 in the control and ≤8.2 μg/L in the 4-NP treatment. In the 17.7-μg/L treatment, however, more females than males were identified, and the sex ratio of males to females was about 1:2 (Table 5).

Induction of testis-ova in the gonads of the F<sub>1</sub> generation was observed at lower 4-NP concentrations than at those in the F<sub>0</sub> generation. In the 8.2- and 17.7-μg/L treatments, testis-ova were observed in two (10%) and five (25%) among 20 fish examined, respectively (Table 5). However, all these fish with testis-ova clearly displayed external male characteristics.

The degree of development of oocytes in each testis-ova specimen was not as severe as that in the F<sub>0</sub> medaka in the 17.7-μg/L treatment, and developing spermatids and spermatozoa were observed in the gonads with testis-ova. In the 4.2-μg/L treatment and the controls, no testis-ova were observed in any of the F<sub>1</sub> medaka examined. A significant difference was observed between the sex ratios obtained from histological examination of the pooled controls and of the fish in the 17.7-μg/L treatment (*p* = 0.002).

### DISCUSSION

Our study clearly demonstrates that 4-NP exerts numerous estrogenic and nonestrogenic effects on each life stage of medaka, depending on the concentrations tested. In our study, the embryo survival and hatching success of the F<sub>0</sub> medaka were significantly reduced only at the highest 4-NP concentration (183 μg/L). However, a 4-NP concentration as low as 17.7 μg/L affected the mortality of the F<sub>0</sub> medaka after swim-up. Although our study found no effect of 4-NP on the growth of the F<sub>0</sub> medaka at 60 d posthatch (even in the 51.5-μg/L treatment), the sex ratio estimated from secondary sex characteristics was completely skewed toward female at a concentration of 51.5 μg/L. Furthermore, gonadal histology revealed that abnormal sexual differentiation, as shown by the presence of oocytes in the testis (testis-ova), occurred in the 17.7- and 51.5-μg/L treatments. These results show that 4-NP affects the gonadal development and survival of medaka at similar concentrations in juveniles. In the reproductive phase, the mean fertility of the breeding pairs was reduced (although not statistically significant) in the 17.7-μg/L treatment. Overall, these results indicate that the LOEC and NOEC of 4-NP throughout

Table 5. Cumulative mortality, growth, and sex ratios as determined by gross examination of secondary sex characteristics and by gonadal histology at 60 d posthatch of F<sub>1</sub> medaka

4-Nonylphenol concn. (μg/L) <sup>a</sup>	<i>n</i>	Mortality (%) <sup>b</sup>	<i>N</i>	Growth		Sex ratio	<i>N</i>	Gonadal histology		
				TL (mm) <sup>c</sup>	BW (mg) <sup>d</sup>	(♂:♀)		Number of fish		
								Testis	Ovary	Testis-ova
Control	60	1.7	59	26.5 ± 1.4	169 ± 38	28:31	20	7	13	0
Solvent control	60	10	54	26.6 ± 1.8	174 ± 40	26:28	20	11	9	0
4.2	60	10	54	26.9 ± 1.5	177 ± 35	25:29	20	9	11	0
8.2	60	18.3	49	27.0 ± 2.2	178 ± 46	24:25	20	10	8	2
17.7	30	6.7	28	25.9 ± 1.9	171 ± 38	9:19	20	4 <sup>e</sup>	11 <sup>e</sup>	5 <sup>e</sup>

<sup>a</sup> Mean measured concentrations.

<sup>b</sup> Cumulative mortality at 60 d posthatch.

<sup>c</sup> Total length.

<sup>d</sup> Body weight.

<sup>e</sup> The sex ratio obtained from gonadal histology differed significantly from that of the pooled controls (*p* = 0.002).

the complete life cycle of the F<sub>0</sub> medaka were 17.7 and 8.2 µg/L, respectively.

In the progeny generation, we observed no concentration-related effect of 4-NP on hatching success in embryos, mortality, behavior, appearance, or growth of hatched larvae. However, we could demonstrate an effect on sexual differentiation at 60 d posthatch: Histological examination of the gonads showed that 10% of fish in the 8.2-µg/L treatment and 25% in the 17.7-µg/L treatment had testis-ova. This result suggests that exposure to 4-NP beginning in ovo and continuing throughout the early life stage of the F<sub>1</sub> generation induced the formation of hermaphroditic gonads at lower concentrations than in the F<sub>0</sub> generation. Two explanations for this finding are possible. First, 4-NP may exert enhanced effects to the progeny generation through maternal transfer of 4-NP into the F<sub>1</sub> embryos, although threshold concentrations in the other endpoints, such as mortality, did not increase. Second, transgenerational exposure to 4-NP may potentiate its estrogenic response by increasing the number of estrogen receptors or by enhancing the affinity of the estrogen receptor of the progeny generation. Metcalfe et al. [35] reported a significantly greater induction of hepatic vitellogenin in male medaka exposed to DDT by maternal transfer compared with that in control males when all males were subsequently exposed to 17β-estradiol (12 mg/L) at 10 months old. However, our study neither determined the concentrations of 4-NP in the spawned eggs nor measured the number of estrogen receptors in the gonads of the F<sub>1</sub> medaka. Because we observed a low incidence (10%) of testis-ova in F<sub>1</sub> medaka exposed to 8.2 µg/L, we cannot substantiate this possible enhanced response without further study. We will investigate in further studies whether the progeny after transgenerational exposure to 4-NP suffer reproductive impairment as adults at lower concentrations than those that caused reproductive impairment in the parental generation.

It has been reported that 4-NP exerts lethal toxicity in fish at levels of tens to hundreds of micrograms per liter. Gray and Metcalfe [23], using a static assay, reported that the embryolarval median lethal concentration (LC50) of *p*-NP for medaka was 460 µg/L. Our study demonstrated that flow-through exposure of medaka to 183 µg/L 4-NP caused embryological abnormality and swim-up failure in the F<sub>0</sub> medaka, resulting in significant mortality. The reason for these different values in embryo-larval toxicity is probably due to differences in stability of 4-NP in the static exposure system used by Gray and Metcalfe, which did not maintain the nominal concentrations [23]. The embryological toxicity of another related alkylphenol substance, octylphenol, has also been examined in medaka and caused embryological abnormalities, including slowed heart rate, blood circulation, and swim bladder inflation, in hatched larvae at the nominal concentrations ≥500 µg/L [36]. In our study, we observed the same symptoms in embryos at 8 d postfertilization in the 183-µg/L treatment. Therefore, it appears that toxicity values of alkylphenols, including 4-NP, range from about 200 to 500 µg/L for the early life stage of fish. Some subacute toxicity tests with fish have shown that 4-NP is lethal to hatched larvae and juveniles at concentrations lower than the previously mentioned embryological toxicity. For example, the LOEC in survival of fathead minnow (*Pimephales promelas*) for 33-d early-life-stage exposure was 14 µg/L [24]. In our study, the mortality of F<sub>0</sub> medaka was increased by prolonging exposure beyond 30 d posthatch, resulting in significant mortality at 4-NP concentrations ≥17.7 µg/L, compared with the pooled controls. This

time-dependent mortality may result from the bioaccumulation of 4-NP, which can accumulate in the lipid tissues of exposed fish [37]. The bioconcentration factors for NP in fish range widely from <1 to 1,250 (average 300), depending on fish species [21,24]. Therefore, prolonged exposure might be required to accumulate a sufficient threshold concentration of 4-NP in the fish body, culminating in delayed mortality of the juvenile medaka. Schwaiger et al. [38] reported that exposure of juvenile carp (*Cyprinus carpio*) to NP (1–15 µg/L) over a 70-d period caused severe anemia. Therefore, chronic exposure of NP might affect general health status by inducing hematological alterations in the fish, leading to reduced survival.

Alkylphenolic substances have been also reported to affect the sexual differentiation of fish. Gimeno et al. [39] exposed genetically male common carp to 4-*tert*-pentylphenol and then observed the formation of oviducts in the gonads. Hermaphroditic gonads (testis-ova) in medaka are induced by treatment with 4-*tert*-octylphenol [40,41]. In our study with 4-NP, testis-ova were observed at concentrations ≥17.7 µg/L, in agreement with the results of a previous *p*-NP study by Gray and Metcalfe [23]. Although the mechanism of the development of testis-ova with these alkylphenols in medaka is unknown, the abnormal development in the gonads might be induced in a concentration-dependent manner by the estrogenic activity of these chemicals. Jobling et al. [21] observed a dose-dependent elevation of plasma vitellogenin levels, accompanied by a reduction in testicular weight, in male rainbow trout exposed to alkylphenol polyethoxylates, including NP. Our results also showed that the incidence and severity of testis-ova in the gonads of F<sub>0</sub> medaka were increased in a concentration-dependent manner. In F<sub>0</sub> fish in the 17.7-µg/L treatment, the incidence of testis-ova was not high (20%), and numerous spermatozoa were observed in the gonad of hermaphroditic fish, indicating active spermatogenesis. These fish still exhibited male secondary sex characteristics. In the 51.5-µg/L treatment, however, 40% of the fish had testis-ova, but none had external male characteristics. This completely skewed sex ratio suggests that the occurrence of testis-ova in this study is due to abnormal sexual differentiation in genetically male medaka exposed to 4-NP. The degree of intersexuality in the gonads of some hermaphroditic fish exposed at 51.5 µg/L was more severe than that at 17.7 µg/L. In specimens from fish exposed to 51.5 µg/L, almost the whole area examined was composed of oocytes, accompanying small testicular tissues interspersed with few spermatocytes and spermatozoa, suggesting inhibition of spermatogenesis. The magnitude of the effects of 4-NP on the sexual differentiation of medaka would depend on the concentrations of 4-NP in the water and probably that in the fish body, and the intersex condition of the gonads in medaka might influence spermatogenesis and subsequently reproductive function.

In our study, the fertility of paired medaka was reduced at a 4-NP concentration of 17.7 µg/L, although it was less pronounced. The GSI of male medaka at the end of reproductive phase was also reduced in the 17.7-µg/L treatment, some of which had testis-ova in the gonads. The GSI reductions have already been found in adult male trout exposed to 4-NP, accompanied by a dose-dependent elevation of plasma vitellogenin levels [21]. Therefore, our results suggest that 4-NP may inhibit testicular development in fish through its estrogenic properties, inhibiting maturation of the testis and potentially reducing fertility in male fish. Gonadal histology of male medaka at 60 d posthatch in the 17.7-µg/L treatment revealed

hermaphroditism, although spermatogenesis was still observed. The development of the intersex condition might depend on the duration of exposure. Gray et al. [40] reported that exposure of medaka to octylphenol at 100  $\mu\text{g/L}$  from hatching for a period of one or two months did not induce testis-ova, but exposure for three months resulted in 3 of 50 males developing this condition. Gimeno et al. [42] also reported that exposure of sexually mature male carp to 4-*tert*-pentylphenol for three months resulted in the progressive disappearance of spermatozoa and spermatogenic cysts with increasing exposure time. Shibata and Hamaguchi [43] exposed sexually matured male medaka to 17 $\beta$ -estradiol (160  $\mu\text{g/L}$ ) for 30 d and then observed the increasing development of testis-ova and inhibition of spermatogenesis in the testes with increasing duration of exposure. Therefore, in our study, prolonged exposure (for 60 d posthatch) of male medaka with testis-ova may have preceded oogenesis, as well as deficient sperm quality and quantity. The reduction in both fertility and GSI in male medaka exposed to 17.7  $\mu\text{g/L}$ , although not significant, indicates that the reproductive abilities of these fish may have been affected by the estrogenicity of 4-NP and suggests that further examination of this response is needed. On the other hand, exposure to 4-NP (4.2–17.7  $\mu\text{g/L}$ ) had no effect on fecundity of female medaka and enlarged their ovarian size (as indicated by the GSI of spawning females in our study). This indicates that 4-NP did not have adverse effects on the reproductive abilities of female medaka at the concentrations tested. Nimrod and Benson [44] reported that the fecundity of medaka exposed to NP at 0.5, 0.8, or 1.9  $\mu\text{g/L}$  from hatch to one month did not change among treatment groups and suggested that this factor would not be compromised by larval exposure to NP.

Several monitoring studies on NP levels in aquatic environments have generally shown concentrations less than 1  $\mu\text{g/L}$ , but in some monitoring sites NP levels exceed 10  $\mu\text{g/L}$ . A survey of rivers in the United Kingdom found <10  $\mu\text{g/L}$  in most rivers examined, and only one river had up to 180  $\mu\text{g/L}$  [12]. In the United States, NP concentrations in rivers and STW effluents are generally lower than those found in the United Kingdom; a survey of 30 U.S. rivers found that 70% of the sites had concentrations of 0.1  $\mu\text{g/L}$  or less [13]. Snyder et al. [14] reported that NP was detected in 17 of 23 samples of effluents and surface waters collected from several locations in the Trenton Channel of the Detroit River, with concentrations ranging from <0.011 to 37  $\mu\text{g/L}$ . Hale et al. [15] detected NPs at concentrations of <1.0  $\mu\text{g/L}$  in 20% of the 59 effluents from sewage treatment plants in Virginia, USA, and concentrations of >10  $\mu\text{g/L}$  in 10% of effluents. One treated-effluent water sample from Cape Cod (MA, USA) contained NP at 15.9  $\mu\text{g/L}$  [16]. In Japan, the Environmental Agency surveyed NP levels in various rivers and estuaries and reported that NP was less than 1.1  $\mu\text{g/L}$  in all the 42 samples examined [17]. The LOEC of 4-NP obtained in our study (17.7  $\mu\text{g/L}$ ) was at least one order of magnitude greater than the ambient levels of NP present in most rivers and effluents. However, our study suggests that 4-NP may affect wild fish populations in some sites in which it is detected at >10  $\mu\text{g/L}$ . Furthermore, Routledge et al. [4] reported that exposure to 10 ng/L of 17 $\beta$ -estradiol elevated plasma vitellogenin levels in the male rainbow trout but not in the male roach, indicating species differences in sensitivity toward estrogen exposure. Therefore, the life-cycle toxicity of NP in other fish species remains to be investigated.

In summary, this study demonstrates that life-cycle expo-

sure of 4-NP to medaka causes lethal toxicity and also affects adversely its sexual differentiation and reproduction as shown by the induction of hermaphroditic gonads and reduced fertilization rates, respectively. Overall, this study establishes a LOEC and NOEC of 4-NP through the life cycle of the  $F_0$  medaka of 17.7 and 8.2  $\mu\text{g/L}$ , respectively. In the progeny generation ( $F_1$ ) exposed to those concentrations of 4-NP, no significant effects were observed on hatching success, post-hatch mortality, or growth, whereas sexual differentiation at 60 d posthatch was affected. An intersex condition of the gonads in the  $F_1$  fish was induced not only in the 17.7- but also in the 8.2- $\mu\text{g/L}$  treatment, suggesting the enhanced effect of 4-NP on sexual differentiation in the progeny generation compared with  $F_0$ , by continuous exposure from beginning in ovo to the juvenile stage.

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