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Toxic Effects in *Siganus oramin* by Dietary Exposure to 4-*tert*-Octylphenol

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Abstract The integrated toxicities of 4-*tert*-octylphenol (*t*-OP) on *Siganus oramin* were investigated by dietary administration at doses of 5, 25 and 125 mg/kg body weight over 28 days. Significant increase was observed in the activity of hepatic glutathione *S*-transferase at 125 mg/kg on both day 14 and 28 in males, and at all doses on day 28 in females, and in hepatosomatic index at 25 mg/kg on day 14 in both sexes. Plasma levels of testosterone and cortisol decreased significantly at all doses on day 28. Histopathologic changes in liver, spleen, intestine and testis deteriorated with increasing doses and duration. The results suggest that *S. oramin* is sensitive to *t*-OP, and the above endpoints may be potential biomarkers for evaluating toxicities of environmental pollutants such as *t*-OP.

Keywords 4-*tert*-Octylphenol · *Siganus oramin* · Toxic effects · Biomarkers

Alkylphenol polyethoxylates (APEOs) are an important group of non-ionic surfactants commonly used in household detergents, industrial detergents and tanneries, as well as in paints, herbicides, and many other formulated products (Isidori et al. 2006). In nature, APEOs degrade to alkylphenols such as 4-nonylphenol (NP), 4-octylphenol (OP) and 4-*tert*-octylphenol (*t*-OP). In the last few years, the environmental safety of APEOs has given rise to great concern. Available data showed that alkylphenols displayed high toxicities, especially estrogenic activity to affect hormone production, gonadal development and spermatogenesis in mammals (Bendsen et al. 2001), and to induce vitellogenin production or decrease gonadal weight in teleost fish (Madsen et al. 2006). Alkylphenols are stable and easily accumulated in the environment, especially in aquatic ecosystems, and may be bioaccumulated in fish tissues and tissues of other aquatic organisms with a bioconcentration factor from several hundreds to more than one thousand (Isidori et al. 2006). It was reported that alkylphenols and their ethoxylates were found in all eight analyzed edible marine species (two crustaceans and six fish species) from the Adriatic Sea (Italy), from which the daily intakes for these compounds were estimated to be about 0.1–12 μg/day for an Italian adult living along the Adriatic Coast (Ferrara et al. 2005). This finding suggests that alkylphenol toxicities may be passed and biomagnified from aquatic organisms to terrestrial animals, even to humans. Thus, the systematic study of alkylphenol toxicity on fish can not only be of significance for the protection of fishery resources, but also provide evidence of the potential effects of alkylphenols on human health via the food chain. Until now, however, integrated investigation of toxic effects of alkylphenols has been seldom conducted in fish.

Rabbitfish are distributed widely in the coral reefs of the Indo-Pacific region, among which the euryhaline *Siganus oramin* is a commercially important species along the coast of Southern China. Its omnivorous nature means the fish is exposed to pollutants not only directly from contaminated water, but also from the food chain. It is possible to use this species as a potential indicator organism for marine environmental contaminants including *t*-OP, and it has been used similarly for heavy metal pollution (Lai et al. 1999). In present study, multitiered biological responses, including biochemical, physiological and cytological parameters were investigated to evaluate *t*-OP's toxic effects on

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S. oramin. The results may provide some potential biomarkers for evaluating toxicities of *t*-OP on fish, and also present data for evaluating this fish as a potential sentinel organism in coastal and estuarine environments.

Materials and Methods

Sexually immature *S. oramin* weighing 15–20 g were caught in autumn 2005 from an unpolluted coast near Nan Ao Marine Biology Station (NAMBS) of Shantou University, Southern China. After rearing in an indoor seawater cement pool for more than 1 month at NAMBS, the fish were acclimated to laboratory conditions for 2 weeks prior to experiments in a continuous flow-through system consisting of forty aquariums (size 50 × 60 × 80 cm, volume 240 L) supplied with filtered running seawater. Each aquarium was stocked with 15–20 fish. Aquarium water was kept oxygen-saturated by aeration and its temperature changed with room temperature (18–22°C). Water salinity was 32 ± 1 ppt, and photoperiod was 12-h light:12-h dark. Fish were fed twice daily with commercial fish pellets (FengHuang, Brand, China) at a total of 3% body weight (BW) per day.

The experiment was conducted in November 2005 and consisted of three *t*-OP exposed groups and one control group, each with triplicate aquariums. One day prior to the exposure, fish of approximately the same size were pooled into a plastic bucket, and then randomly distributed in 12 aquariums, each containing 15 fish with an average weight of 19.09 g. Their sexes were indistinguishable by visual observation.

t-OP (purity >90% by GC, Fluka Co., Switzerland) was administered to fish via the food. At the beginning of the experiment, three kinds of *t*-OP-containing food were prepared in one batch and kept at −20°C. Based on the BW of fish, feeding ratio and experimentation duration, 0.6 kg of *t*-OP-containing food was prepared for each group. First, 100, 500 and 2,500 mg of *t*-OP were separately dissolved in absolute ethanol, and then were respectively mixed with 0.6 kg commercial fish pellets. After ethanol evaporation, the nominal *t*-OP concentrations were 0.5, 2.5, and 12.5 mg per 3 g food, respectively. In the *t*-OP exposed groups, fish received *t*-OP-containing food every second day at a total of 3% BW in daily two meals, which resulted in 5, 25, and 125 mg/kg BW exposure doses, respectively; on the other days, they received food with no *t*-OP as in the control group at a total of 3% BW. The experiment was maintained for 28 days, and fish care was continued as described above. No fish died during the entire course of experiment.

On day 14, seven fish were randomly sampled from each tank. The remaining fish were sampled on day 28. Fish were anaesthetized with 0.01% 2-phenoxyethanol and blood was collected from the caudal vein using heparinized syringes and the #7 gauge needles. Blood samples were held at 4°C for processing. Fish were weighed and subsequently killed by spinal transection. Livers were quickly removed and weighed, and then snap-frozen in liquid nitrogen and stored at −80°C for enzymatic activity analysis. Additionally, samples of liver, spleen, anterior intestine and gonad were fixed in Bouin's solution (4°C) for 12–24 h, dehydrated in a graded ethanol series and embedded in paraffin. Sections (4–6 μm) were stained with haematoxylin and eosin (H & E) and observed under light microscopy.

Plasma was separated from the blood samples by centrifugation at 1,650*g* for 15 min at 4°C; a proteinase inhibitor (aprotinin, 156 KU/mL) was then added to the plasma, which was stored at −80°C pending hormone measurement. Since *t*-OP was shown to have estrogenic effects in vivo in fish and other vertebrate species, plasma steroids (testosterone and cortisol) were measured only for males with a chemiluminescence immune detection system (ACS: 180SE) and corresponding test kits from Bayer Corporation Diagnostics Division, USA.

Enzyme extracts from liver tissues and the measurements of glutathione *S*-transferase (GST) activity were conducted using commercial test kit (Nanjing Jiecheng Bioengineering Institute, China) according to the manufacturer's instruction. Briefly, tissues were homogenized (10% W/V) using 0.1 M Tris–HCl buffer (pH 7.4, EDTA 0.1 mmol/L, NaCl 0.8%). After the homogenates were centrifuged at 1,200*g* for 15 min at 4°C, the remaining supernatant was used for the measurement of protein content and GST activity with the kits. The activity of soluble GST was determined by colorimetry using 1-chloro-2,4-dinitrobenzene and glutathione (GSH) as substrates, and was calculated in terms of the protein content of the sample. It is reported in units per milligram of protein (U/mg protein), where 1 unit is the conversion of 1 μmol/L of GSH per min at 37°C. Each enzymatic assay was carried out in triplicate.

All data are presented as means ± SEM. Differences between the control and *t*-OP exposed groups were analyzed by one-way ANOVA followed by Tukey's test. Significance was set at $p < 0.05$. Data for males and females were individually analyzed. Hepatosomatic Index (HSI) was calculated by LW/BW × 100%, where LW and BW were liver weight and body weight, respectively.

Results and Discussion

After *S. oramin* were exposed to *t*-OP for 14 or 28 days, a series of toxic effects were observed. At the biochemical level, significant increases in hepatic GST activity were

found at 125 mg/kg exposure on both day 14 and 28 in males, and at all doses on day 28 in females (Table 1). GST is a multi-component enzyme involved in the detoxification of electrophilic compounds during phase II metabolism by conjugation with glutathione in liver (Jokanovic 2001); it has a crucial role in defense against oxidative damage (Monteiro et al. 2006). Thus the increase of GST may be a physiological response for detoxification or increase of free radical species after *t*-OP exposure. As for the gender-related responses in GST activity, similar results were reported in Nile tilapia *Oreochromis niloticus* upon exposure to paraquat (Figueiredo-Fernandes et al. 2006). The hepatic GST activities in both control and *t*-OP exposed groups on day 28 were lower than those on day 14 (Table 1). This may be due to the lower average water temperature at the end of experiment compared with the earlier time. Similarly, GST activity displayed seasonal variations and some positive dependence on water temperature in juvenile rainbow trout and mature eelpout (Ronisz et al. 1999).

The physiological data showed that significantly lower plasma levels of testosterone and cortisol were found in all the *t*-OP exposed groups on day 28 (Table 2); a significant increase in HSI was found at 25 mg/kg on day 14 (Table 3). The endocrine control of steroid production in teleost fish is very complex. The decrease of plasma steroids may be due to increased catabolism, decreased production, or a combination of these factors. OP is an agonist of the pregnane X-receptor and constitutive androstane receptor, both of which are capable of regulating several detoxification enzymes such as those of the CYP2B and CYP3A families responsible for the metabolism of steroids (Kretschmer and Baldwin 2005). *t*-OP may affect the synthesis of testosterone through regulating the hypothalamo-pituitary-gonad axis or blocking hydroxylase activity (Hanioka et al. 2000). Additionally, the structural damage of testis (one of the main steroidogenic tissues) by *t*-OP as described below (Fig. 1h) may also explain the decrease of plasma steroids. HSI is generally considered an indicator of estrogenic effects. The increase of HSI observed in this study is consistent with *t*-OP's estrogenic effects as reported in teleost fish (Madsen et al. 2006). HSI did not increase with exposure doses or duration, and this may be due to the hepatic damage described below (Fig. 1b).

At the cytological levels, histopathological changes in liver, spleen, intestine and testis of *S. oramin* exposed to *t*-OP were observed and appeared to be related to both dose and duration of exposure. After 14 days exposure, hepatic lesions could be observed in the 5 mg/kg BW group as the hyperplasia of Kupffer cells and congestion, and in the 25 and 125 mg/kg BW groups as the hepatocellular vacuolization and necrosis (Fig. 1b). These morphological changes were aggravated on day 28. Splenic changes included the congestion in the peripheral tissue in 25 and 125 mg/kg BW groups after 14 days; at 28 days,

Table 1 Hepatic glutathione *S*-transferase activity (U/mg protein) in *Siganus oramin* after orally administered 4-*tert*-octylphenol for 14 or 28 days

Exposure days	Sex	4-*tert*-Octylphenol doses (mg/kg BW)			
		0 (Control)	5	25	125
14	Females	225.68 ± 13.12	259.76 ± 8.03	249.75 ± 6.82	239.39 ± 19.90
	Males	230.69 ± 10.14	235.08 ± 13.31	264.34 ± 13.34	319.63 ± 13.57**
28	Females	150.24 ± 9.13	194.14 ± 9.73*	199.27 ± 16.29*	214.00 ± 9.31**
	Males	172.38 ± 7.60	167.40 ± 6.68	209.47 ± 19.17	219.67 ± 8.83*

Note: Values are expressed as Mean ± SEM (n = 7–8). For values in each row, * $p < 0.05$ and ** $p < 0.01$ indicate significant difference from control by one-way ANOVA followed by Tukey's test

Table 2 Plasma testosterone and cortisol levels in *Siganus oramin* males after orally administered 4-*tert*-octylphenol for 14 or 28 days

	Exposure days	4-*tert*-Octylphenol doses (mg/kg BW)			
		0 (Control)	5	25	125
Testosterone (μg/L)	14	1.80 ± 0.09	1.74 ± 0.10	1.85 ± 0.06	1.88 ± 0.09
	28	1.9 ± 0.19	1.47 ± 0.06*	1.60 ± 0.06*	1.57 ± 0.10*
Cortisol (μg/dL)	14	5.39 ± 2.17	5.89 ± 2.95	5.48 ± 1.89	5.86 ± 2.61
	28	6.70 ± 2.04	1.11 ± 0.61**	0.49 ± 0.23**	0.28 ± 0.09**

Note: Values are expressed as Mean ± SEM (n = 4–6). For values in each row, * $p < 0.05$ and ** $p < 0.01$ indicate significant difference from control by one-way ANOVA followed by Tukey's test

Table 3 Hepatosomatic index (%) in *Siganus oramin* after orally administered 4-*tert*-octylphenol for 14 or 28 days

Exposure days	Sex	4-*tert*-Octylphenol doses (mg/kg BW)			
		0 (Control)	5	25	125
14	Females	0.93 ± 0.03	0.92 ± 0.03	1.15 ± 0.06**	0.94 ± 0.04
	Males	0.94 ± 0.02	0.97 ± 0.04	1.14 ± 0.06**	0.92 ± 0.03
28	Females	0.97 ± 0.04	0.96 ± 0.15	0.98 ± 0.05	0.94 ± 0.03
	Males	0.95 ± 0.07	0.93 ± 0.04	0.93 ± 0.05	0.95 ± 0.05

Notes: Values are expressed as Mean ± SEM (n = 7–8). For values in each row, ** $p < 0.01$ indicates significant difference from control by one-way ANOVA followed by Tukey's test

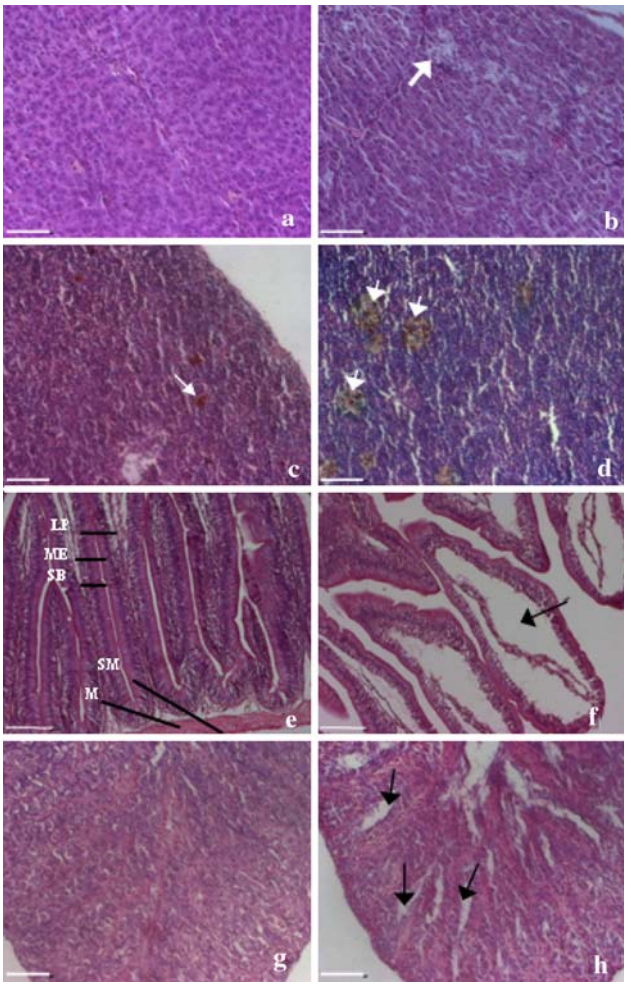

Fig. 1 Light micrographs of liver (**a**, **b**), spleen (**c**, **d**), intestine (**e**, **f**) and testis (**g**, **h**) from control or 4-*tert*-octylphenol exposed *Siganus oramin* at 25 or 125 mg/kg body weight for 14 or 28 days. (**a**, **c**, **e**, **g**): control. (**b**), 125 mg/kg, 14 d; (**d**, **f**, **h**), 125 mg/kg, 28 d. White arrows indicate hepatocellular necrosis in (**b**), congestion of blood vessels in (**c**) and hemosiderosis in (**d**), respectively; black arrows indicate vacuoles in (**f**) and (**h**). LP, Lamina propria; ME, Mucosal epithelium; SB, Microvilli; SM, Serous membranes; M, Muscularis. Scale bars: 50 μm in (**a–d**, **g**, **h**), 100 μm in (**e**, **f**). H & E, ×200 for (**e**, **f**), ×400 for others

congestion appeared in the 5 mg/kg BW group, and lots of hemosiderosis appeared in the 25 and 125 mg/kg BW groups (Fig. 1d). Intestinal lesions were not obvious after 14 days' exposure; at 28 days, however, histological lesions including necroses, dissolution and vacuolization of intestinal mucosal epithelium appeared in the 25 and 125 mg/kg BW groups (Fig. 1f). Obvious histopathological changes were observed in the testis, but not in the ovary. On day 14, lesions of testis tissues could be observed in some individuals in 125 mg/kg BW group; at 28 days, lesions including the dissolution and vacuolization of spermatocytes became more obvious in all the *t*-OP exposure groups (Fig. 1h).

These observed histological lesions are all readily explainable relative to the *t*-OP toxicity. The liver is an important organ for the biotransformation and excretion of xenobiotics, and environmental pollutants may alter hepatic biochemistry, physiology and structure (Thophon et al. 2003). The morphological changes of liver corresponded with the elevated hepatic GST activity, possibly related to the increased free radical production from the metabolism of *t*-OP. Free radicals are known to attack macromolecules including lipid, DNA and protein, and cause apoptosis and necrosis. In the normal spleen tissues, hemosiderosis is not widespread. In adverse or contaminated environments, however, macrophages may engulf decrepit erythrocytes and break down haemoglobin, producing hemosiderosis (Pacheco and Santos 2002). Intestinal damage was not unexpected since *S. oramin* were exposed to *t*-OP via ingestion. The testicular lesions caused by *t*-OP exposure were similar to the reported toxic effects of estradiol and OP on sperm production in guppy *Poecilia reticulata* (Kinnberg et al. 2003).

In conclusion, the present study showed that *t*-OP at 5–125 mg/kg BW can pose multiple adverse effects on *S. oramin*, including the increase of hepatic GST activity, decrease of plasma testosterone and cortisol levels, and obvious histopathological changes in liver, spleen, intestine and testis. The pattern of toxicity noted in this investigation is somewhat related to dose and duration of exposure. These results suggest that the above endpoints may be used as biological parameters for evaluating the toxic effects of *t*-OP on marine fish. Based on these results, *S. oramin* has the potential to be used as a sentinel organism for contaminants in coastal and estuarine environments.

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