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Effects of 4-tert-octylphenol on glutathione-related antioxidant status in olive flounder *Paralichthys olivaceus*

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Effects on glutathione-related antioxidant parameters were examined after a chronic exposure of olive flounder, *Paralichthys olivaceus* to dietary 4-*tert*-octylphenol (4-*tert*-OP). Fish were fed diets containing 4-*tert*-OP at 0, 1, 5 and 10 mg/kg diet for 6 weeks. Antioxidant parameters examined were reduced glutathione (GSH) contents and enzyme activities of glutathione reductase (GR), glutathione S-transferase (GST) and glutathione peroxidase (GPx) in tissue homogenates of the liver, kidney and gill. It was observed that all parameters examined increased although there were some differences in dose responses and temporal patterns in the increase. GSH contents increased after exposure to 4-*tert*-OP in the three organs examined. However, the GSH increase was evident only after 4 weeks in the liver whereas it was elevated after 2 weeks in the kidney and gill. GR activity exhibited a significant increase in response to 4-*tert*-OP at 1 mg/kg in all three organs, however, its activity returned to control levels when exposed to 5 and 10 mg/kg. Hepatic GST activity showed an earlier increase at week 2 in contrast to the kidney and gill where they increased after 4 weeks of 4-*tert*-OP exposure. Temporal patterns in GPx activity changes to 4-*tert*-OP exposure were dissimilar among the organs: hepatic activity increased from week 2 through week 6; renal activity increased transiently at week 2; gill levels were higher through weeks 4 - 6. The results suggest that elevation of several GSH-related antioxidant parameters can be considered as evaluation criteria for 4-*tert*-OP-induced oxidative stress in a fish.

Key words: Glutathione-related parameters, 4-*tert*-Octylphenol, Olive flounder *Paralichthys olivaceus*, Oxidative stress

Introduction

Alkylphenol polyethoxylates (APEOs) account for the major class of non-ionic surfactants and have a variety of industrial and commercial applications. Octylphenol ethoxylates are one of two most abundant APEOs in the marketplace (Talmage, 1994). These APEOs when discharged into the aquatic environment are bio-degraded to produce octylphenols (OPs). One of the OPs products is 4-*tert*-octylphenol (CAS No. 140-66-9) depending on their parent compound structures.

The metabolite OPs are more toxic than the parent compound (Naylor *et al*., 1992) and has been demonstrated to exist in seawater, river water and sewage wastewater (Ferguson *et al*., 2000; Meesters and Schroder, 2002; Petrovic *et al*., 2002; Staniszewska

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et al., 2014). 4-*tert*-OP is a potent estrogen in vitro and in vivo (Beresford *et al*., 2000; Yoshida *et al*., 2001). The compound is known to cause reproductive perturbation (Gray *et al*., 1999), apoptotic cell death (Toomey *et al*., 1999), endocrinal malfunction (White *et al*., 1994) and vitellogenin-like pathologic anomalies (Folmar *et al*., 2001; Masden *et al*., 2003). In particular, 4-*tert*-OP is stronger as an estrogen-mimetic than nonylphenol, another most common APEO metabolite, in rainbow trout (Jobling *et al*., 1996).

Antioxidant status in organisms has frequently been employed in assessing toxic potential of various environmental toxicants (Halliwell and Gutteridge, 1999). Glutathione (GSH) is an important endogenous antioxidant molecule of low molecular weight in an organism (Forman *et al*., 2008). GSH levels can vary in response not only to the existence of pro-oxidant chemicals but also by enzyme activities that synthesize or utilize the molecule. While glutathinoe reductase (GR) produces reduced form GSH by regenerating from the oxidized one (GSSG, glutathione disulfide), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) consume GSH by utilizing it as a substrate. Thus, both intracellular GSH level and these GSH-modulating enzymes reflect antioxidant potential of a cell.

There is only limited number of studies in which effects of 4-*tert*-OP were examined for antioxidant status in animals (Kim *et al*., 2003; Aydoğan *et al*., 2008). The aim of the present study was to investigate whether GSH-dependent antioxidant parameters will be affected in olive founder *Paralichthys olivaceus* when chronically exposed to 4-*tert*-OP. Olive flounder is a very common and widespread teleost species living in coastal zones and estuaries of Korea, Japan and China's seawaters. In the wild habitat the fish hides in sediment and feeds on benthic invertebrates. The species is also one of the most widely cultured fishes in the same area. Because of such abundance, they are very likely to be exposed to APEO metabolites from water and prey as well.

Materials and Methods

Experimental fish

Cultured olive flounder, *Paralichthys olivaceus* were obtained from a local fish farm in Pohang, Kyongbook Province, Korea. Prior to exposure, fish were held for three weeks for acclimatization and evaluation of fish health under laboratory conditions maintaining at 20 ± 2℃ under 12:12 h light/dark cycle. During acclimatization fish were fed a basal diet (Table 1) twice daily (2% body weight for each meal at approximately 10:30 and 16:30 h). After acclimatization, fish of similar sizes (mean body weight, 49.7 ± 3.9 g, mean ± S.D.) were selected for exposure experiments.

Diet preparation

The basal diet (Table 1) was supplemented with 4-*tert*-OP at levels of 0, 1, 5 and 10 mg/kg diets.

Table 1. Composition of the basal diet

Ingredients	g/Kg diet
Casein, vitamin free	335
Gelatin	75
Corn starch	280
Dextrin	140
Squid liver oil	50
Soy bean oil	30
Carboxymethyl cellulose	30
Protease	5
Cellulose	15
Vitamin premix[1]	10
Mineral premix[2]	30

[1]Vitamin mix supplemented diets (in mg/Kg diet): vitamin A (500,000 IU/g), 8; vitamin D_3 (1,000,000 IU/g), 2; vitamin K, 10; vitamin E, 100; thiamine, 10; riboflavin, 20; pyridoxine, 20;vitamin C, 50; nicotinic acid, 150; folic acid, 10; vitamin B_{12}, 0.02; biotin, 2; inositol, 400; choline chloride, 2,000; panthothenate, 200.

[2]Mineral premix contains (in g/Kg premix): NaCl, 43.3; $MgSO_4 \cdot H_2O$, 136.6; $NaH_2PO_4 \cdot 2H_2O$, 86.9; KH_2PO_4, 239.0; $Ca(H_2PO_4)_2 \cdot H_2O$, 135.3; $ZnSO_4 \cdot 7H_2O$, 21.9; Fe-citrate, 29.6; Ca-lactate, 303.89; $AlCl_3 \cdot 6H_2O$, 0.15; KiO_3, 0.15; Na_2SeO_3, 0.01; $CuCl_2$, 0.2; $MnSO_4 \cdot H_2O$, 2.0; $CoCl_2 \cdot 6H_2O$, 1.0.

For this, 4-*tert*-OP was first dissolved in ethyl alcohol (Sigma-Aldrich, St. Louis, MO, purity >97.0%) before mixing with a Hobart mixer. The moist mixture was extruded through a 3-mm diameter extrusion module. The resulting moist pellets were freeze-dried to moisture content of about 10%. Pellets were crumbled to particles, sieved to obtain appropriate sizes and stored at -20℃ until fed.

Experimental design and exposure regime

After acclimatization, ten olive flounder were placed in triplicate 150 L-aquaria (20 ± 2℃) for each dose in seawater supplied with air to maintain dissolved oxygen levels greater than 75% saturation throughout the exposure period. The test aquaria were supplied with continuous flow-through water (flow = 8 L/min). Seven fish from each exposure concentration were sampled for analysis at 2, 4 and 6 weeks after exposure commencement and anesthetized with buffered MS-222 (Sigma-Aldrich, St. Louis, MO) for sacrifice.

Glutathione (GSH) content and GSH-related enzyme activities

Liver, gill and kidney were isolated from the fish and homogenized in four to five volumes of ice-cold TRIS buffer (50 mM, 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, pH 7.5) with several passes in a homogenizer (099C K4424, Glas-Col, Terre Haute, IN, USA). The homogenate was centrifuged (12,000 × g for 15 min, MIKRO 22R, Hettich, Germany) at 4℃, and the supernatant was used for antioxidant parameter assays. Reduced glutathione (GSH) concentration was measured by the spectrophotometric method of Vandeputte *et al*. (1994). Glutathione reductase (EC 1.6.4.2) activity was measured at 340 nm with a slight modification of Goldberg and Sparner (1987). The reaction mixture containined 50 mM imidazole, 0.3 mM EDTA-2Na, 0.1 mM NADPH, and 2 mM oxidized glutathione GSSG (pH 7.1). Glutathione-S-transferase (EC 2.5.1.18) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig *et al*., 1974). Briefly, the reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 1 mM glutathione, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and tissue homogenate equivalent to 20 μg tissue protein. After 5 min reactions at room temperature, absorbance was determined at 25℃ with a spectrophotometer (Shimadzu PC-1601, Kyoto, Japan) at 340 nm. The activity of glutathione peroxidase (EC 1.11.1.9) was kinetically measured at 37℃ from absorbance changes at 340 nm based on the oxidation of NADPH (Beutler, 1984). Total protein concentration was determined using the method of Bradford (1976) with bovine serum albumin as a standard. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and working solutions were prepared on the day of use.

Statistical analysis

Statistical analysis was performed using the SPSS/PC+ statistical package (SPSS Inc, Chicago, USA). Data are expressed in mean ± S.E. Significant differences among groups were determined using one-way ANOVA and Duncan's test for multiple comparisons (Duncan, 1955). Comparison was made among groups of the same exposure periods of the different 4-*tert*-OP exposure concentration. The level of statistical significance was set at $P < 0.05$.

Results

Contents of reduced form glutathione (GSH) in three organs are shown in Fig. 1. Levels in the liver stayed unchanged at week 2, but they tended to elevate after 4 weeks. However, in the kidney and gill, GSH elevation was noted at week 2. When elevated, the increments were similar regardless of 4-*tert*-OP concentrations exposed.

Analytical results for glutathione reductase (GR) activity are presented in Fig. 2. The general pattern of GR changes was similar in all three organs: fish

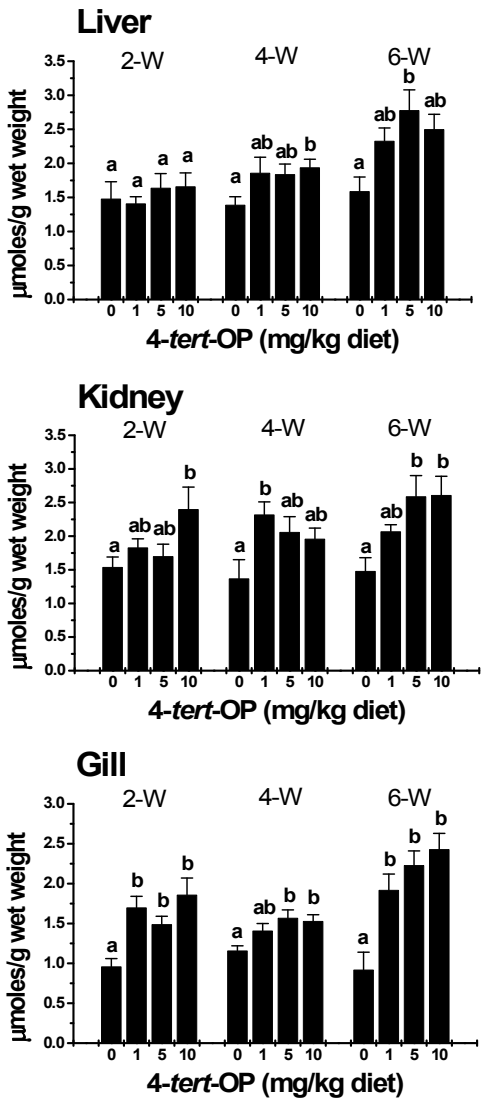

Fig. 1. Glutathione content (μmoles/g wet weight) in liver, kidney and gill of olive flounder exposed to different levels of 4-*tert*-octylphenol for 2, 4 and 6 weeks. Data are represented as mean ± S.E. (n =7). Columns with the same letter are not significantly different (P > 0.05). Statistical comparison was made only within the groups of the same exposure period.

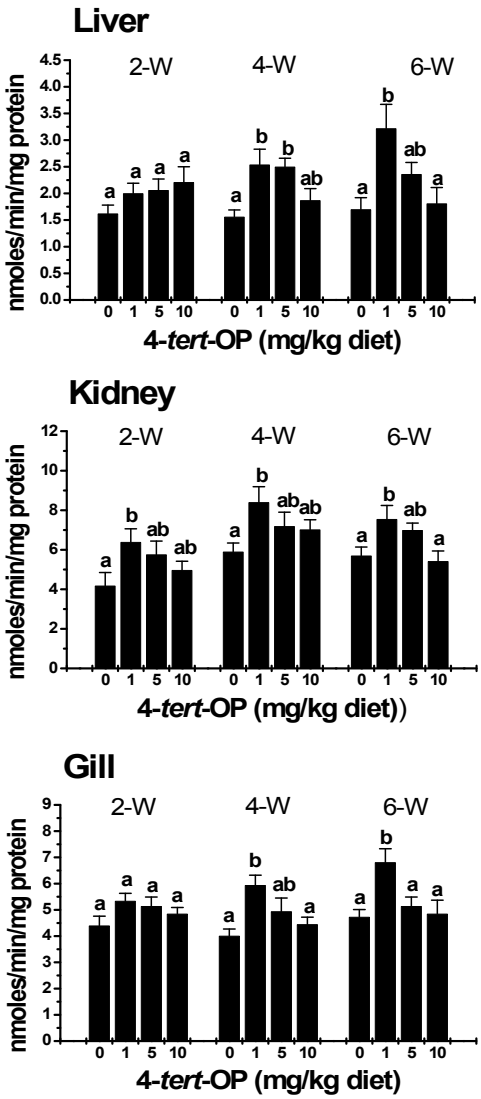

Fig. 2. Glutathione reductase enzyme activity (nmoles/min/mg protein) in liver, kidney and gill of olive flounder exposed to different levels of 4-*tert*-octylphenol for 2, 4 and 6 weeks. Data are represented as mean ± S.E. (n = 7). Columns with the same letter are not significantly different (P > 0.05). Statistical comparison was made only within the groups of the same exposure period.

exposed to 1 and 5 mg/kg showed higher enzyme activities but there was no increase at 10 mg/kg. All such GR increments were apparent only after 4 weeks in the liver and gill. However, GR activity increased earlier to 4-*tert*-OP exposure in the kidney where the response appeared after 2 weeks.

Fig. 3 shows the change in glutathione S-transferase (GST) activity of the control and treated groups. In the liver, elevation of GST was marked from after 2 weeks and there was no further elevation by pro-

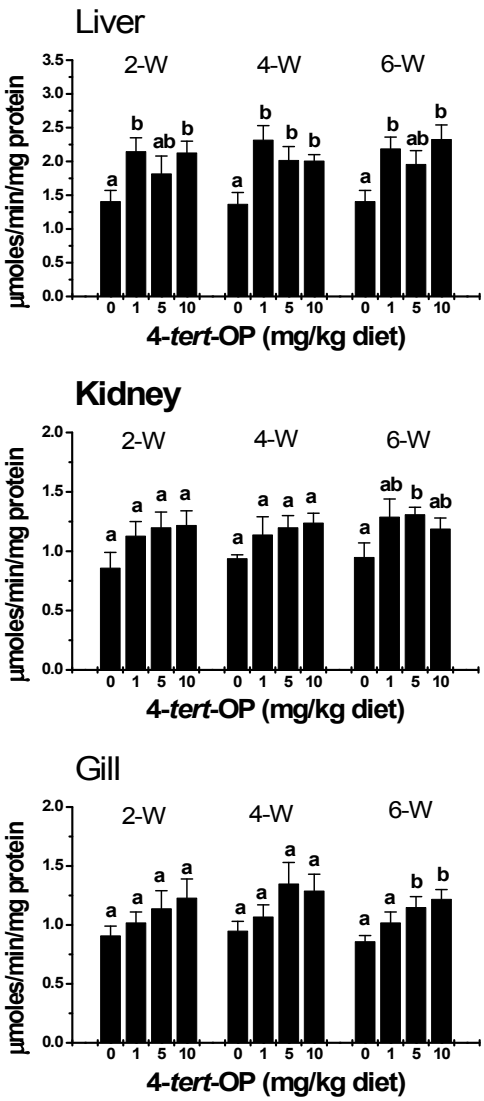

Fig. 3. Glutathione S-transferase enzyme activity (µmoles/min/mg protein) in liver, kidney and gill of olive flounder exposed to different levels of 4-*tert*-octylphenol for 2, 4 and 6 weeks. Data are represented as mean ± S.E. (n = 7). Columns with the same letter are not significantly different (P > 0.05). Statistical comparison was made only within the groups of the same exposure period.

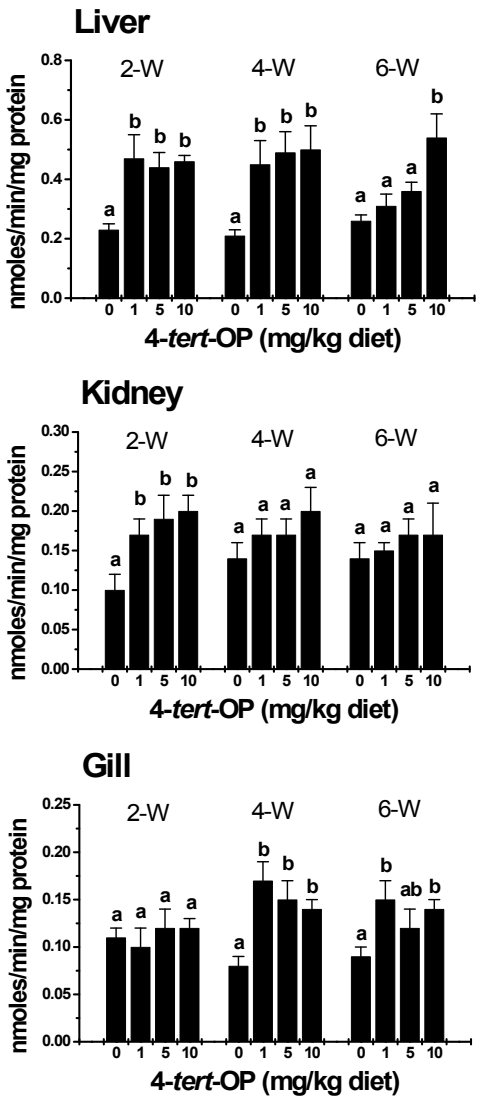

Fig. 4. Glutathione peroxidase enzyme activity (nmoles/min/mg protein) in liver, kidney and gill of olive flounder exposed to different levels of 4-*tert*-octylphenol for 2, 4 and 6 weeks. Data are represented as mean ± S.E. (n = 7). Columns with the same letter are not significantly different (P > 0.05). Statistical comparison was made only within the groups of the same exposure period.

longing exposure period up to 6 weeks. In addition the elevation level was not different among the three exposure concentrations. The responsiveness in GST elevation was less pronounced in the kidney and gill, in which organs the elevation was noticed only after 6 weeks.

Glutathione peroxidase (GPx) activities after 4-*tert*-OP exposure are shown in Fig. 4. In the liver, GPx

levels approximately doubled over the control level during the 2 - 6 weeks of exposure. This increase, however, seemed to disappear in 1 and 5 mg/kg exposure at week 6. Kidney GPx elevated earlier at week 2, but the elevation was not recognizable thereafter. On the other hand, gill GPx enzyme activity was higher during week 4 through 6 in fish exposed to 4-*tert*-OP at all tested concentrations.

Discussion

Organisms have developed antioxidant defense mechanisms to minimize oxidative cellular damages imposed by chemical toxicants (Filho, 1996; Winston and Di Giulio, 1991; Gadagbui and Goksøyr, 1996). The failure of an organism to operate such defense mechanisms enough to scavenge excess free radicals can result in significant cellular damage and the condition so-called oxidative stress occurs (Halliwell and Gutteridge, 1999). Numerous environmental chemicals have been dealt in studies for their impact on antioxidant parameters in various organisms including fishes. In particular, glutathione (GSH) and GSH-regenerating/utilizing enzyme systems work in a close cooperation in order to handle most of the oxidative stresses derived from chemical exposure (Winston, 1991). However, almost nothing is known about the effects of 4-*tert*-OP as an oxidative stressor and roles of GSH-related systems in fish.

Cellular glutathione (GSH) participates in the metabolism and detoxification of xenobiotics and thus has been the theme of many investigations (Ross *et al*., 1986; Stein *et al*., 1992; Doroshow, 1995). Tissue levels of reduced GSH can be increased or decreased depending the balance between its synthesis and consumption: an adaptive mechanism to oxidative stress will increase its synthesis, but a severe oxidative stress may decrease GSH levels due to overwhelming oxidation to oxidized form, GSSG (Forman *et al*., 2009). In this study, we observed that GSH levels in all three tissues increased after 4-*tert*-OP exposure. The temporal pattern in GSH increase was not identical among tissues, however. Relative slow rise in the liver compared with kidney and gill may indicate that there could be an operation of reduced GSH elevation mechanisms, either net GSH synthesis or accelerated reduction of already present molecules, but not to the extent to exceed GSH consumption. The *de novo* synthesis of GSH is mediated by two consecutive enzyme systems, γ-glutamylcysteine synthetase and glutathione synthetase, incorporating precursor amino acids (Halliwell and Gutteridge, 1999). As we did not directly assess those synthetic enzyme activities, it's not clear yet whether 4-*tert*-OP mediated induction of the synthesis enzymes.

Quite often, pro-oxidant stressors reduce or deplete GSH contents by excess utilization, thus the decrease in the contents is considered as an indicator for oxidative stress. However, the present result shows that 4-*tert*-OP caused a significant increase of GSH content in liver, kidney and gill of treated fish. The increase may be interpreted as the evidence that 4-*tert*-OP activated GSH synthesis system but its utilization rate was far less than its production resulting in net elevation. Although 4-*tert*-OP is a pro-oxidant (Aydoğan *et al*., 2008), GSH depletion seem to be well compensated by an accelerated production. Similarly to our finding, a fungicide chlorothalonil stimulated increase in GSH contents in the liver, gill and kidney of channel catfish, *Ictalurus punctatus* (Gallagher *et al*., 1992). Elevation in GSH was also observed in fishes exposed to contaminated field samples (Di Giulio *et al*., 1993; Chen *et al*., 1998).

Glutathione reductase (GR) catalyzes reduction of oxidized glutathione (GSSG) to the reduced form GSH in an NADPH-dependent coupling reaction (Ou *et al*., 1996), maintaining homeostasis in GSH/GSSG levels (Winston and Di Giulio, 1991). After chronic exposure in flounder, glutathione reductase (GR) enzyme activity increased in response typically to low concentrations of 4-*tert*-OP in the three tissues. An increased GR activity was observed in laboratory experiments

with fish exposed to PCBs (Rudneva-Titova and Zherko, 1994; Otto and Moon, 1995), PAHs (Tjärnlund *et al*., 1996) and hexaclorobenzene-contaminated food (Roy *et al*., 1995). It was also demonstrated in a field study that halogenated xenobiotics induced hepatic GR activity in the liver of Baltic salmon (Pesonen *et al*., 1999). This increase was also shown in shorthorn sculpin caught from PAHs polluted harbors (Stephensen, 2000). The increase in reduced GSH content, discussed above, however, may not be explained simply by an increase in GR enzyme, which changed in opposite direction to GSH elevation at high concentrations of 4-*tert*-OP. High levels of 4-*tert*-OP may cause GR inhibition rather than stimulation or induction. Thus, it seems that 4-*tert*-OP should have certain mechanisms to enhance GSH synthesis itself yet to be expolored.

GST enzyme conjugates electrophilic metabolites to facilitate their excretion from aquatic animals (Dierickx, 1984; Stenersen *et al*., 1987). When the liver, kidney and gill of flounder were examined, we observed that GST enzyme activities were markedly elevated in the liver from week 2 to all concentrations of 4-*tert*-OP exposure. Similar elevation of the same enzyme activity was reported for fish exposed to chlorothalonil (Davies, 1985) and to benzo(a)pyrene (Fair, 1986). The alteration in GST activity to 4-*tert*-OP reflects stressed status in olive flounder, and the immediate response suggests expression of the GST enzyme mRNA as reported in a fish after 4-*tert*-OP treatment (Yu *et al*., 2008). Hepatic GST activity assessment may be suggested as a sensitive tool for biomonitoring oxidative stress to this chemical.

Glutathione peroxidase (GPx) metabolizes peroxides such as hydroperoxides and hydrogen peroxide, and protects cell components from lipid peroxidation via a glutathione-dependent mechanism (Behne and Wolters, 1983). GPx activity can be induced strongly by a variety of environmental pollutants (Lackner, 1998). We observed that GPx activity in the liver and kidney increased from week 2, while its activity was slowly elevated in the gill. Furthermore, the maintenance of heightened activity was not consistent in different tissues indicating that complicated induction dynamics are involved for GPx enzymes. The liver is a major site of detoxification and the first target of ingested pro-oxidants. It is considered to be the most important tissue in the cellular protective role against lipid peroxidation induced by xenobiotic materials. In such processes, GPx and GR both being abundant in fish tissue play important roles (Di Giulio *et al*., 1993).

The current study was aimed at assessing oxidative stress by 4--*tert*-OP examining its effects on glutathione-dependent enzyme activities and glutathione content in the liver, kidney and gill. Overall results indicate that 4--*tert*-OP elevates a battery of glutathione-related antioxidant parameters possibly through induction of involved enzymes. However, the responses were tissue specific in both sensitivity and temporal variations. The results of the study should also be considered as a contribution to the establishment of biomarkers for 4--*tert*-OP -induced oxidative stress in fish.

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