J. Appl. Ichthyol. 32 (2016), 636–642

ISSN 0175–8659

*Received: July 21, 2015*
*Accepted: August 28, 2015*
doi: 10.1111/jai.13072

# The effects of endosulfan on *P450* 1A gene expression, antioxidant enzymes activity and histopathological alterations in liver of Persian sturgeon (*Acipenser persicus* Borodin, 1987)

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**Summary**

The effects of 14 days exposure to sublethal concentrations of endosulfan (10 and 40 $\mu g\ L^{-1}$) were investigated in mRNA- *P450* 1A expression, antioxidant enzymes (SOD and CAT) activity and histopathological alterations of Persian sturgeon (*Acipenser persicus*) fingerlings with weights of 3–5 g. The results illustrated that the relative mRNA- *P450* 1A expression level significantly increased ($P < 0.05$) compared to the control group. Highest significant increase ($P < 0.05$) was observed on the first day, then decreased towards day 14 of exposure. The SOD and CAT activity showed a significant increase in fish exposed to different concentrations up to day 7, then activity decreased on day 14 in fish of all treated groups. Although signs of tissue lesions were observed on day 4, they increased from day 7 and reached the highest level on day 14. The magnitude of all changed studied parameters (gene expression, enzymes and histopathological) follows a concentration-dependent manner.

## Introduction

Among all forms of chemical pesticides, organochlorines are considered to be the most hazardous with respect to environmental pollution, since they are very persistent, non-biodegradable and capable of bio-magnification as they move up in the food chain (Dong et al., 2013). Endosulfan (6,7,8,9,10,10-Hexachloro-1,5,5a,6,9,9a-hexahydro- 6,9-methano-2,4,3-benzodioxathiepine-3-oxide), an organochlorine, is widely used in agriculture as well as in integrated agriculture-aquaculture farming systems to protect important food crops (Dong et al., 2013; Piazza et al., 2015). According to the USA environmental protection agency (EPA), endosulfan has deleterious effects on the health of aquatic organisms at concentrations above 0.22 $\mu g\ L^{-1}$ (acute) and 0.056 $\mu g\ L^{-1}$ (chronic) (Mersie et al., 2003). Following its classification in Annex A of Stockholm Convention on Persistent Organic Pollutant, it will be withdrawn from the global market by 2020 (Dong et al., 2013). At present, 60 countries are phasing it out yet despite the application limitation of endosulfan in many countries, it is still widely used in most developing countries because of its effectiveness and low cost of application (Ondarza et al., 2014). Even exposure of fish to low concentrations of this pesticide can accumulate in the body and have numerous toxic effects that include tissue damage, physiological, biochemical and molecular alterations, respiratory changes, and ultimately death, all of which can be used as environmental bio indicators (Dar et al., 2015; Piazza et al., 2015). The intensity and duration of these responses are influenced by several factors, including the concentration of the toxicant, duration of exposure, and the species of fish (Piazza et al., 2015). Changes at the molecular and biochemical level are usually the first detectable responses to environmental perturbation, which can provide information on the sublethal cellular effects of stressors in a particular species of interest, and have the potential for application as sensitive biomarkers in field studies to monitor fish health (Kim et al., 2009). Among the most commonly used biochemical biomarkers, those related to oxidative stress are assumed to have an important position, being used frequently in both environmental monitoring and laboratory assays (Pandey et al., 2003). In fact, the metabolism of xenobiotics is a two-phase process. The first-phase reactions include oxidation, reduction and hydrolysis in which the greatest importance is ascribed to oxidation enzymes and in which the most important oxidation enzymes of this phase are P450 cytochromes (Lewis, 2001). In the second phase reactions, endogenous enzymatic and non-enzymatic antioxidants converse reactive oxygen species (ROS) to harmless and also protect and restore normal cellular metabolisms and functions (Rastgoo and Alemzade, 2011). The key enzymes for detoxification of ROS in all organisms are superoxide dismutase, glutathion-s- transferase and catalase (Ballesteros et al., 2009). Nowadays, changes of the amount of mRNA – *P450* 1A induced by xenobiotics could be considered as a biomarker, indicating the pollution of aquatic environment (Dong et al., 2013). Shao et al. (2012) stated that endosulfan was highly genotoxic in *Danio rerio* due to the significant level of DNA damage estimated at concentrations of 0.01 $\mu g\ L^{-1}$. Damage to protein and lipid structure of cell membrane can *per se* cause tissue damage (Glover et al., 2007). Persian sturgeon (*Acipenser persicus*) is one of the most economically important fishes in the Caspian Sea, in which its stock have declined dramatically mainly due to over fishing, illegal catch, pollution and deterioration of

habitats and natural spawning grounds (Pourkazemi et al., 2000; Yarmohammadi et al., 2014). Numerous studies have been conducted on histopathological and biochemical effects of some types of pollutant on fishes (Safari et al., 2014); however, studies on endogenous enzymatic antioxidants and molecular responses to contaminants (toxicogenomic) are limited (Safari et al., 2014). Thus, the present study was performed to investigate the sublethal effects of endosulfan exposure in *A. persicus* using endogenous enzymatic antioxidans, the histopathological and molecular parameters of the liver.

## Materials and methods

Persian sturgeon (3–5 g) fingerlings were obtained from the Shahid Marjani Breeding and Rearing Center (Golestan, Iran) and acclimated to experimental conditions for a period of 15 days, after which fish were randomly distributed into nine 300-L tanks at a density of 30 fish per tank and submitted to sublethal concentrations of endosulfan 35%, using a mixture of α- and β- isomers (70 : 30), 10 and 40 $\mu$g $L^{-1}$ for 14 days, based on the LC 50 of 80 $\mu$g $L^{-1}$ reported by Safari et al. (2015). A stock solution was prepared by dissolving the technical-grade endosulfan in acetone (final solvent concentration of 0.001%). Three replicate tanks were considered for each treatment. During the exposure, fish were fed with live food (Artemia biomass) twice a day and the water was continuously monitored for temperature, dissolved oxygen, pH and conductivity daily with a water checker (HORIBA U-10, Japan) (mean ± SD, T ~ 24 ± 1 °C; DO ~ 7 ± 0.2 $mgO_2$ $L^{-1}$; pH ~ 7.6 ± 0.2;1412 ± 167.9 Ms $cm^{-1}$). Two-thirds of the water was renewed every 24 h by adding endosulfan stock solution to minimize endosulfan loss and to reduce contamination of tanks after feeding. Actual endosulfan concentrations were measured in water samples 30 min after renewal by gas chromatograph- electron capture detector (Environmental Protection Agency SW846 M8081A, US Environmental Protection Agency, 1996), Water samples were extracted using neutral methylene chloride. The extraction solvent was exchanged to hexane. The extracts were cleaned with florisil. Two microlitre aliquots of the pesticide extracts and a pesticide standard mixture were injected for analysis, resulting in 9.5 $\mu$g $L^{-1}$ (for nominal 10 $\mu$g $L^{-1}$) and 39 $\mu$g $L^{-1}$ (for nominal 40 $\mu$g $L^{-1}$).

### Sampling

Three fish per replicate (nine fish per treatment) were taken at days 0, 4, 7 and 14, and rapidly anesthetized with clove powder (0.5 g $L^{-1}$). The livers were removed from the samples and immediately deep-frozen in liquid nitrogen and frozen at −80 °C until the begin of the experiment.

### Molecular analysis

**RNA isolation and cDNA synthesis.** Total RNA isolation was done following BIOZOL Reagent protocol (Bioflux-Bioer, China). The concentration of RNA samples was evaluated by Nanophotometer (IMPLEN-P100) reading at 260/280 nm and their integrity verified by ethidium bromide staining of 28 S and 18 S ribosomal RNA (rRNA) bands on non-denaturing agarose gel (1.5%). DNA in samples was removed by treatment with *DNase* I (Fermentas, France). RNA of three samples of each replicate was then pooled (Yarmohammadi et al., 2014); 1 $\mu$g of total RNA was used to synthesize first-strand cDNA using a Fermentase cDNA Synthesis kit for RT-PCR, following the manufacturer's instructions and a mixture of oligo- dT as primer.

**Primer design.** The qPCR primers for the amplification of the partial segment of *A. persicus P450* 1A were designed from the homologous regions of the sequences of *Acipenser ruthenus* (JN 5647452.2) and *Acipenser schrenckii* (JX0139352.2) using (Primer3: http://frodo.wi.mit.edu/primer3). The qPCR efficiency was also taken into account for selection of the best qPCR primer pair and correct size. β-actin was used as a housekeeping gene, since it had been previously validated by Safari et al. (2014). The sequences of primers, melting temperature and product length are listed in Table 1.

**Quantitative real-time PCR (qPCR).** Real-time PCR analysis was carried out using an iCycler (BioRad) with SYBR Green qPCR Master Mix (1×) and all primers at [300 nM] after the protocol suggested by Safari et al., 2014. The fold change in *P450* 1A relative mRNA expression was calculated by the $2^{-\Delta\Delta C_t}$ method. The obtained data were analyzed using the IQ5 optical system software version 2.0 (BioRad). A melting curve analysis was performed after every amplification program to verify specificity of target and the absence of primer dimers and a no template control (NTC) was included with each assay to verify that PCR master mixes were free of contamination. To ensure that PCR conditions were optimal, a $\log_{10}$ dilution series was produced from undiluted cDNA pooled together from randomly selected treatments, used to generate a standard curve. The standard curve was used to estimate efficiency ($E$) and reproducibility of the assay and run in triplicate on each PCR. Reproducibility was represented by the $R^2$ value of the standard curve and was greater than 0.95.

Table 1
Name, sequence, melting temperature (Tm) and product length of primers used in the present study to quantify *P450* 1A transcript of Persian sturgeon *Acipenser persicus* through Real-time PCR

| Primer name | Primer sequence | Tm | Product length |
|---|---|---|---|
| Ap p450q-PCRF | GTCATCTGTGCCATGTGCTT | 56 | 237 |
| Ap p450q-PCRR | TCTTGTCGAAGGAGCGGTAG | 56 | |
| β-actin q-PCRF | TTGCCATCCAGGCTGTGCT | 56 | 215 |
| β-actin q-PCRR | TCTCGGCTGTGGTGAA | 56 | |

#### Oxidative stress analysis

**Sample preparation.** Liver was homogenized 1:5 in ice-cold 50 mM phosphate buffer pH 7.5 containing protease inhibitor cocktail (sigma p2714). The homogenate was centrifuged at 10 000 ***g*** for 10 min at 4 °C and the supernatant was kept at −80 °C for antioxidant enzyme activities assays. Protein content was assayed by the method of Lowery et al. (1951).

#### Antioxidant enzymes activity

**Superoxide dismutase (SOD).** The SOD activity was measured by its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT), as described by Dhindsa et al. (1981). The activity of SOD was expressed as nanokatal per milligram protein.

**Catalase (CAT).** The CAT activity was assayed spectrophotometrically by measuring the $H_2O_2$ decomposition in time at 240 nm according to the Aebi (1984). The enzyme activity was expressed as nanokatal per milligram protein.

**Histological examination.** Liver samples of the control and exposed fish were dissected and fixed in 10% neutral-buffered formalin; the samples were then processed for routine wax histological evaluation (dehydrated and embedded in paraffin). Five micrometre sections were prepared and stained with hematoxylin and eosin stain, as described by Haschek et al. (2010).

#### Statistical analysis

Relative gene expression was calculated by the Pfaffl formula (Pfaffl et al., 2002). The ratio between the target (*P450* 1A) and housekeeping (β-actin) genes was analyzed by the REST software (Pfaffl et al., 2002). A Kolmogorov–Smirnov test was used to assess for normality of distributions for both gene expression and oxidative enzymes data. Normalized data passed the Levene's test for homogeneity of variance. Statistics data were subjected to one-way ANOVA with $\alpha = 0.05$. Comparisons within each analysis day and within a treatment at different sampling days were performed by Duncan's test. Data are reported as mean ± standard deviation (X ± SD). SPSS software ($\alpha = 0.05$), version 16 (SPSS, Richmond, VA), was used.

## Results

#### Gene expression

The endosulfan exposure significantly increased *Ap P450* 1A expression in the liver of Persian sturgeon. In 10 $\mu$g $L^{-1}$, the relative expression of *Ap P450* 1A significantly increased compared to the control, reached the highest level (4.25-fold, $P < 0.05$) on day 1 of exposure, then decreased. This decreasing trend was not significant until day 4 (3.5-fold, $P > 0.05$) and on day 7 (3.1-fold, $P < 0.05$) and day 14 (2.5-fold, $P < 0.05$). A similar trend (5.5-fold, $P < 0.05$) on day 1, (4.9-fold, $P > 0.05$) on day 4, (4.2-fold, $P < 0.05$) on day 7 and (3.77-fold, $P < 0.05$) on day 14 were also observed in 40 $\mu$g $L^{-1}$ (Table 2). *Ap P450* 1A mRNA expression significantly increased in fish treated with 40 $\mu$g $L^{-1}$ then 10 $\mu$g $L^{-1}$ of endosulfan (Table 2).

#### Enzymes assay

The SOD and CAT enzyme activities in the liver of endosulfan-exposed (10 and 40 $\mu$g $L^{-1}$) *A. persicus* generally increased compared to the control group. In all studied concentrations a slight non-significant increase was observed in SOD and CAT enzymes by day 4 of exposure ($P > 0.05$). However, on day 7 the activity of both studied enzymes increased significantly ($P < 0.05$), followed by a non-significantly decrease on day 14 of exposure. The enzyme activity increased significantly in fish treated with endosulfan concentrations of 40 $\mu$g $L^{-1}$ rather than 10 $\mu$g $L^{-1}$ of (Figs 1 and 2).

#### Histopathology

The histopathological observations for both control and treatment groups are summarized and representative images of the liver tissue during the experiment are shown in Fig. 3. The liver of control fish showed normal histology with no morphological changes. On the first day of exposure, the detected histopathological damages were not considerable. On day 4, some structural changes such as massive necrosis, hydropic swelling, nuclear degeneration, hemorrhage, dark granules, apoptic necrosis and dilation of sinusoid were observed. In all treatments the intensity of lesions increased gradually over time, up to day 14.

## Discussion

The response to pollutant exposure in fish depends on the life cycle, habitat, food, biology of each species in addition to other factors (Piazza et al., 2015). By exposure to examined concentrations of endosulfan in this study, neurotoxic effects were evidenced – such as restlessness, and jerky and erratic swimming. Abnormal behavior including irregular swimming, reduced activity, avoidance response and deposition of a slimy-whitish film on the body of the fish exposed

Table 2
Alternative Relative mRNA levels to β-actin activity in liver of Persian sturgeon *Acipenser persicus* exposed to 10 and 40 $\mu$g $L^{-1}$of endosulfan for 14 days

| Exposure day/Concentration | 1 | 4 | 7 | 14 |
|---|---|---|---|---|
| 10 $\mu$g $L^{-1}$ | $4.25 \pm 1^{a}{}_{A}$ | $3.5 \pm 0.31^{ab}{}_{B}$ | $3.1 \pm 0.1^{b}{}_{A}$ | $2.5 \pm 0.22^{b}{}_{A}$ |
| 40 $\mu$g $L^{-1}$ | $5.5 \pm 0.2^{a}{}_{A}$ | $4.9 \pm 0.5^{ab}{}_{A}$ | $4.2 \pm 0.9^{b}{}_{A}$ | $3.77 \pm 0.9^{b}{}_{A}$ |

Results expressed as means with standard deviation (n = 9). Different superscript and subscript letters = significant ($P < 0.05$) difference in each column (A–B) and each row (a–b), respectively.

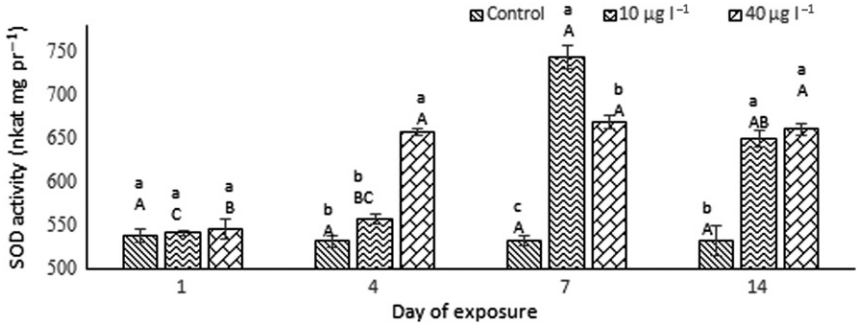


Fig. 1. Alterations in SOD (nkat mg protein$^{-1}$) activity in Persian sturgeon liver exposed to 10 and 40 $\mu$g L$^{-1}$ endosulfan for 14 days. Results expressed as means with standard deviation (n = 9). Different letters denote significant ($P < 0.05$) difference in each exposure day (a–c) and each concentration (A–D), respectively.

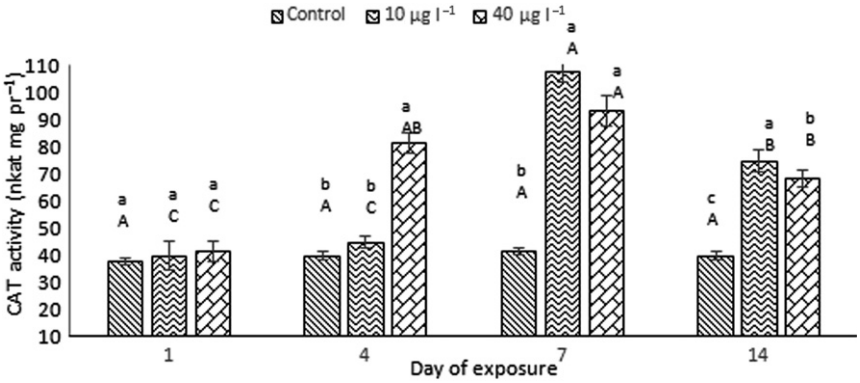


Fig. 2. Alterations in CAT (nkat mg protein$^{-1}$) activity in Persian sturgeon liver exposed to 10 and 40 $\mu$g L$^{-1}$ endosulfan for 14 days. Results expressed as means with standard deviation (n = 9). Different letters characterize significant ($P < 0.05$) difference in each exposure day (a–c) and each concentration (A–D), respectively.

to high concentrations of pollutants, especially in a short period after exposure, was reported by Shariati et al. (2011). Alterations in *P450* gene expression in response to environmental contaminants were observed in a variety of fish species for evaluating the extent to which an organism is stressed (Zhang et al., 2012; Miao et al., 2014). In the present study the transcriptional expression of *P450* 1A showed a clear time-dependent response in the liver after the fish were exposed to endosulfan (Table 2). Although the relative mRNA- *P450* 1A level revealed an increase in all study days compared to the control, a higher up-regulation was observed on the first day. The highest expression on the first day may be attributed to the important role of this enzyme in metabolism of xenobiotics. In fact, the induction of *P450* 1A is mediated by the Ah receptor (AhR). Endosulfan is lipophilic in nature and easily diffuse into cells. The receptor-contaminant complex, transported to the nucleus, resulted in the expression of gene coding for this cytochrome (Billard et al., 2002). It has been demonstrated that the toxicity of a pollutant is related to the degree of its affinity to AhR, and that pollutants with a high binding ability for AhR also have a high capacity to induce *P450* (Billard et al., 2002). *P450* might also be one of the early genes that activate late response genes, which can be considered as another reason for a higher up-regulation on the first day (Waisberg et al., 2003). Liver CYP1 activity in tilapia (*Oreochromis niloticus*) was evaluated through ethoxyresorufin O-deethylase (EROD), with an increase evident immediately after exposure to 0.001 $\mu$g g$^{-1}$ endosulfan (Coimbra et al., 2007). The observed decrease on day 7 may be attributed to the activation of an antioxidant enzyme to detoxify ROS; the reduction on day 14 may be due to gene silencing having arisen from necrosis and histopathological damages to the liver tissues, which *per se* were observed in the present study. In this regard, Dong et al. (2013) suggested that reduction of *P450* after day 7 of exposure could have also been caused by the metabolism products of endosulfan, self-catalyzed routes or by inhibition of CO. Similar studies presented the biphasic response of the *P450* induction by pollutants (Dong et al., 2013). In the present study *Ap P450* 1A mRNA expression increased in higher concentrations and followed a concentration-dependent manner. A contrary result was observed after exposure to high endosulfan levels for longer periods in zebrafish liver and CYP activity was abrogated by the pollutant and its metabolites (Dong et al., 2013). Studies confirmed that the moment, intensity and level of *P450* expression were closely related to xenobiotic, exposure time, concentrations, and species, as well the study tissue (Kim et al., 2009; Huang et al., 2014). Reactive oxygen species (ROS) are induced by substances such as traditional metal ions, pesticides, and petroleum pollutants (Rastgoo and Alemzade, 2011). Free radicals are also produced by endogenous cellular sources during normal cell metabolism, but elevated production of ROS can cause oxidation of protein and lipid, alterations in gene expression, and changes in cell redox status (Wang et al., 2008). Hepatocytes, like other cells, are dependent on antioxidant enzymes for protection against reactive oxygen species produced during the biotransformation of xenobiotics (Dazy et al., 2009). The main antioxidant enzyme system consists of superoxide dismutase (SOD) which detoxifies superoxide

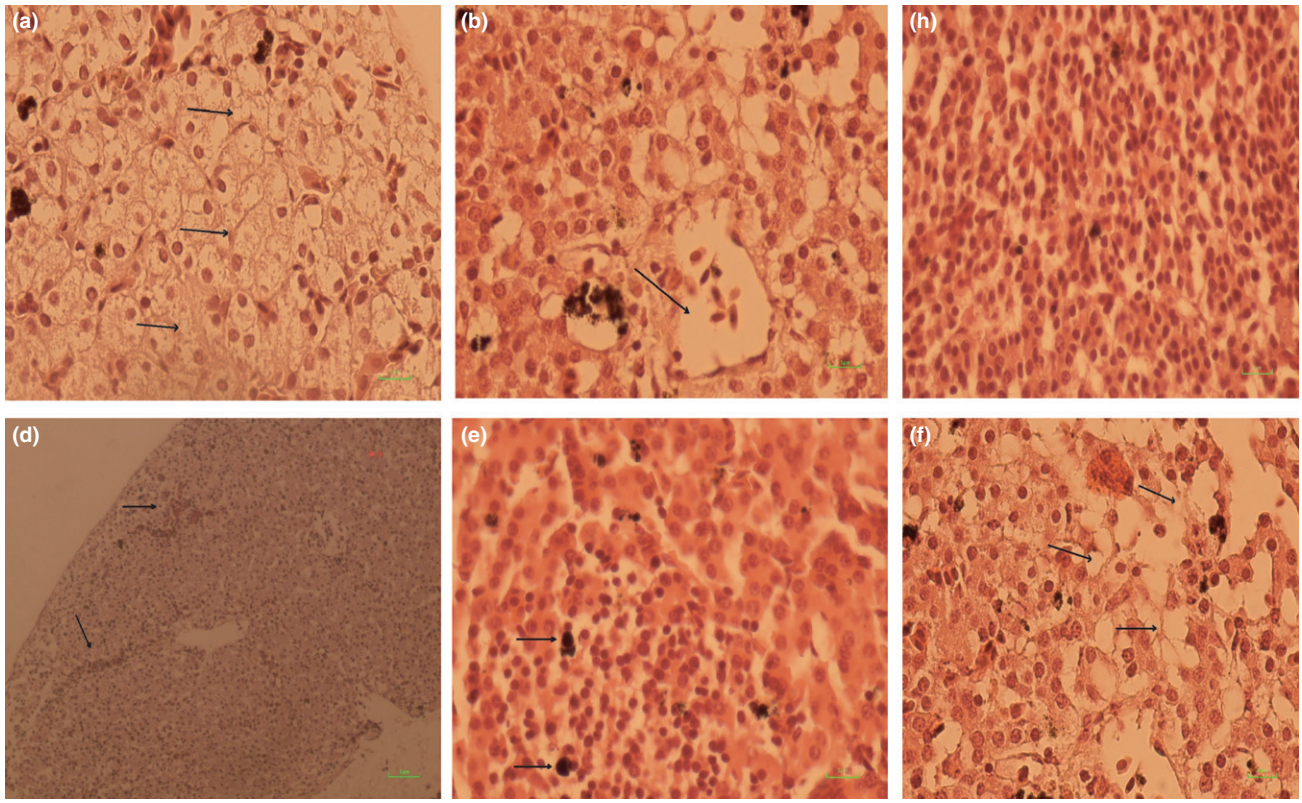


Fig. 3. Histological appearance of Persian sturgeon liver tissue after exposure to endosulfan at 10 and 40 $\mu g\ L^{-1}$ concentrations. (H and E stain): massive necrosis (a), hydropic swelling (b), hemorrhage (d), dark granules (e), apoptic necrosis (f) and control (h).

($O_2^-$) radical into either ordinary molecular oxygen ($O_2$) or hydrogen peroxide ($H_2O_2$) and catalase (CAT), which reduces $H_2O_2$ to $H_2O$ and $O_2$ via two-electron transfer. These enzymes work collaboratively on detoxification of superoxide ions (Dazy et al., 2009; Rastgoo and Alemzade, 2011). In the present study, the increase in SOD and CAT activity until day 7 of exposure (Figs 1 and 2) appears to be probably attributed to superoxide radical accumulation. Similar results were revealed in *Oreochromis mossambicus* (Kumar et al., 2011), *D. rerio* (Crupkin et al., 2013; Dong et al., 2013) and *Penaeus monodon* (Dorts et al., 2009) after exposure to endosulfan and *Jenynsia multidentata* (Monserrat et al., 2014) and attributed to *de-novo* synthesis of enzymatic proteins and induction of the expression of genes encoding SOD and CAT to detoxify ROS. The depressed SOD and CAT activity on day 14 of exposure might also result from the accumulation of endosulfan and its metabolite in the liver, which is considered the main organ for metabolism and the main site for accumulation of pollutants. Our finding is in agreement with the results of Pandey et al. (2003) in the freshwater fish *Channa punctatus,* and Madesto and Martinez (2010) in *Prochilodus lineatus* exposed to endosulfan; those authors reported inactivation of antioxidant enzymes as a result of overproduction of ROS or binding of non-essential heavy metals to the active enzyme site. In fact, the antioxidant enzyme responses to pollutants could be species-specific in addition to factors such as dose and exposure duration (Agnelli et al., 2006; Dorts et al., 2009). In the present study, the enzyme activity increased in higher concentrations and followed a concentration-dependent manner, as the enzyme activity increased significantly in fish treated with 40 $\mu g\ L^{-1}$ rather than 10 $\mu g\ L^{-1}$ of endosulfan. Histopathological indices have been largely used as biomarkers in the monitoring of fish health status during exposure to pollutants such as pesticides, both in experimental and environmental studies (Piazza et al., 2015). Histopathological examination of the livers mainly showed necrosis, hydropic swelling, nuclear degeneration, hemorrhaging, dark granules dilation of sinusoid that could explained by lipid peroxidation, destruction of the cellular membrane and distribution of the activity of ion regulation channels that inhibits intracellular oxidative phosphorylation of hepatocytes (Zaragoza et al., 2000). In this study the intensity of the lesions followed a concentration-dependent manner and increased in the higher doses. Similar studies reported a significant time- and dose-dependent increase in liver structural damages (Da Cuna et al., 2011). These histopathological changes are similar to the observations reported in the liver tissue of *Gambusia affinis, Salmo salar, Onchorhyncus mykiss, Cichlasoma dimerus, Cyprinus carpio, Oreochromis niloticus* and *Trichogaster trichopetrus* after exposure to arsenic, deltamethrin, endosulfan, diazinon, endosulfan, paraquat, paraquat and heavy metals as reported by Cengiz and Unlu, 2003; Glover et al., 2007; Vinodhini and Narayanan, 2009; Da Cuna et al., 2011; Ada et al., 2012; Banaee et al., 2012, 2013, respectively. In conclusion, our data demonstrated that a set of effects was

induced in Persian sturgeon after short-term exposures to a sub-lethal concentration of endosulfan. Up-regulation of *P450* 1A gene and the histopathological injuries were observed in the liver. Moreover, alterations in the antioxidant factors were seen. Based on overall results obtained in this study, the up- and down-regulation of genes may be the first sign of stress that may subsequently be reflected in biochemical and histopathological indicators of toxicity. The magnitude of all studied parameter changes (gene expression, enzymes and histopathological) follows a concentration- and time-dependent manner.

**Acknowledgements**

We would like to express our appreciation to the Gorgan University of Agricultural Science and Natural Resources for their financial support.

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