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ESTIMATION OF ANTIOXIDATIVE ENZYMES IN THE TESTES AND LIVER OF THE *HETEROPNEUSTES FOSSILIS* EXPOSED TO PENTACHLOROPHENOL

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ABSTRACT

Oxidative stress has become an area of significant interest for toxicological studies. Estimation of antioxidative enzymes such as Catalase (CAT), Superoxide dismutase (SOD), Lipid peroxidase (LPO) and Glutathion peroxidase (GPx) have been measured in the fish exposed to 1/10th LC_{50} (32µg/l) PCP for 14 and 28 days. SOD activity and CAT activity in testes and liver were found to be decreased as compared to control groups, whereas levels of Malondialdehyde (MDA) and GPx in testicular and hepatic tissue were significantly elevated as compared to control groups of catfish. Results showed oxidative stress alteration due to PCP exposure. Oxidative damage is produced in response to the exposure to environmental contaminants, by generating free radicals and altering antioxidant enzyme systems. The results of the present investigation indicate that exposure of PCP induces significant changes in the enzymatic profiles in *Heteropneustis fossilis*. The presence of such level of PCP in the natural environment is dangerous to the ecosystem and will definitely affect the survival of fish.

Keywords **:** Antioxidative enzymes, Catfish, *H. fossilis*, Oxidative stress.

Introduction

Extensive use of pesticides finds their way to aquatic environment and becomes a serious problem and affects the health and survival of non-target organism. Oxidative stress is more often used as a biomarker to study the effects of environmental pollution in aquatic environments. Compounds that can inhibit or prevent the oxidation of oxidizable materials by scavenging free radicals and reducing oxidative stress are defined as Antioxidants. Many pollutants may exert toxicity related to oxidative stress. The level of antioxidant enzymes in organism have been extensively taken as an early warning indicator of pollution (Lin *et al.*, 2001). Now days, most environmental problems are attributed to the production and release of toxic chemicals which are capable of interacting with the environment and disrupting the ecosystem. The pentachlorophenol (PCP) known to be a widely distributed persistent environmental contaminant (Heudorf *et al.*, 2000; Ge *et al.*, 2007; Geyer *et al.*, 1987; WHO, 1987; Muir and Eduljee, 1999; OEHHA, 1997). The PCP exposure has been associated with many adverse effects on health and the eco environment (WHO, 1987; Verma and Dubey, 2019, 2021). Based on inadequate evidence in humans but sufficient evidence in animal studies, the International Agency for Research on Cancer classified PCP as a group 2B carcinogen (IARC, 1991). Potential thyroid disrupting effects of PCP have also been revealed (Dallaire *et al.*, 2009; Van den Berg, 1990).

The global restriction on PCP production and use has resulted in reduced PCP contamination in the environment in different world regions (Muir and Eduljee, 1999). In China the primary use of PCP and its sodium salt (Na-PCP) is to kill Schistosome intermediate host snails (Ge *et al.*, 2007; CESE, 2004), wood preservation and other uses account for the secondary (Zhou *et al.*, 2005). The increased PCP use could be resulted in more environmental contamination and potential health risks. In addition to the health risks caused by PCP itself, the impurities of commercial Na-PCP, polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs), can be released into the environment with the use of Na-PCP, this compounds the health risks caused by PCP (Bao *et al.*, 1995; Zheng *et al.*, 2008). Analysis of biomarkers in aquatic organisms particularly in fish is a validated approach for early warning of chemical exposure (Lin *et al.*, 2001; Van der Oost *et al.*, 2003; Osman *et al.*, 2010). It is suggested that during stress conditions fish are capable to change and adapt their metabolic functions (Malarvizhi *et al.*, 2012). The inhibition or induction of the enzymes such as glutamate oxaloacetate transaminases (GOT), glutamate pyruvate transaminase (GPT), lactate dehydrogenase (LDH), acid phosphatase (ACP), alkaline phosphates (ALP) etc., can be used to indicate tissue damage (Nemcsok and Boross, 1982; Webb *et al.*, 2005). Enzymes play important roles in protecting the cell against the potentially toxic effects of the environment (Kuthan *et al.*,

1986), superoxide dismutase catalyzes the dismutation of the superoxide ion (O^{2-}) to hydrogen peroxide and oxygen molecule during oxidative energy processes. GPT, GOT and LDH functions as link enzymes between the protein and carbohydrate metabolism, and also serve as an indicator of chemical stress. Moreover, pesticides enhanced the production of reactive oxygen species (ROS) in aquatic organisms which may lead to impairment of many cellular functions (Uner *et al.*, 2006; Monteiro *et al.*, 2009; Modesto and Martinez, 2010; De Menezes *et al.*, 2011). Antioxidant systems can prevent or counterbalance the deleterious effect of free radicals (Lushchak, 2011; Singh *et al.*, 2011). Fish are reported to possess both mitochondrial and cytosolic superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and non-enzymatic (reduced glutathione and vitamin E) antioxidants as in mammals (Droge, 2002) for detoxification of free radicals (Lushchak, 2011). It is stated that the level of enzyme SOD and CAT can be used as markers of pollutant induced oxidative stress in aquatic organisms (Borkovi *et al.*, 2005). These antioxidants are essential to maintain the redox status of fish cells, likewise, alterations in Lipid peroxidation (LPO) concentration are also used to express severe oxidative damage and are used as a biomarker of effect (Van der Oost *et al.*, 2003; Lackner, 1998). Malondialdehyde (MDA) is a naturally occurring product of LPO which acts as its indicator (Charissou *et al.*, 2004; Pampanin *et al.*, 2005). Fish are obviously subjected to exposure to different pollutants often present in the aquatic environment (Velkova-Jordanoska *et al.*, 2008). Fish has been used as indicator species for monitoring of pollution in the aquatic environment. *H. fossilis*, a catfish, is used for this study to determine the health status by estimating the antioxidative enzymes in the testes and liver of the fish exposed to PCP.

Material and Methods

Experimental setup

Pentachlorophenol (Crystalline, 99% pure) was purchased from Acros organics (Geel, Belgium). Other chemicals used were of analytical grade and purchased locally. Pentachlorophenol was dissolved in ethanol and then diluted with water to obtain the required concentrations 32µg/l($1/10^{th}$ of LC_{50}).

Estimation of LC_{50}

The freshwater male catfish, *H. fossilis* were purchased from the local market in Varanasi and used for the experiment. Estimation of $LC_{50}$for PCP, the protocol of Secretria Estatual Do Meio Ambient (SEMA, 1988) was followed. The LC_{50} value for fish was 320µg/l and for further experiments $1/10^{th}$ LC_{50} (32µg/l) dose were selected (Pandey and Dubey, 2015). Mortality was observed to be 3 in number. They were maintained in the laboratory under normal photo period (13.0L: 11.0D) and temperature (25 ± 2^0c) until use for experiments. The fish were fed with boiled egg daily *ad libitum.* The experiments were performed in accordance with the guidelines for experimentation in animals and all care was taken to prevent cruelty of any kind.

The acclimatized adult male fish classified into two groups (15 fish per each): first group control, second group PCP treated (for 14 days and 28 days with 32µg/l/day). In the present study, the amount of PCP exposures was 32µg/l and the exposure concentrations were determined by performing LC_{50} experiment. The conditions of the experiment were as that of acclimatization with changing all the containers water and concentrations of PCP every day. Male catfish *H. fossilis* (30-45g) were purchased from local fish market in the Resting phase for the experiment.

Measurement of antioxidant status in liver and testes

Estimation of Catalase (CAT) activity

CAT activity was assayed by the method of Chance and Maehly (Chance and Maehly, 1955). The liver and testes were homogenized (10%) in 50 mM phosphate buffer, pH 7.0, and centrifuged at 16,000g for 45 min. The supernatant was used for the enzyme assay. The reaction mixture contained 2 ml of phosphate buffer (pH 7.0), 0.45 ml H_2O_2, and 0.025 ml of enzyme source. The enzyme activity was expressed as micromoles of H_2O_2 metabolized/milligram protein/minute, at 250 nm of absorbance.

Estimation of Super oxide dismutase (SOD) activity

SOD activity was measured as the inhibition of photo reduction of nitrobluetetrazolium (NBT) by the enzyme as per the method of Beauchamp and Fridovich (Beauchamp and Fridovich, 1971). The tissues were homogenized in (10% w/v) potassium phosphate buffer (pH 7.5) containing 1% polyvinyl pyrolidine and centrifuged at 16,000g for 15 min. The supernatant was used as the enzyme source. The total reaction mixture consisted of 100 mM phosphate buffer, 10mM EDTA, 130 mM methionine, 750 mM NBT, 60 mM riboflavin, and enzyme source. The reaction was initiated by the addition of riboflavin, the samples were placed under fluorescence for 30 min, and the resulting color was read at 560 nm against a reagent blank kept in a dark place. The activity was expressed as units/milligram protein.

Estimation of Glutathione peroxidase (GPx) activity

GPx activity was assayed by the method of Paglia and Valentine (Paglia and Valentine, 1967) with modifications according to Lawrence and Burke (Lawrence and Burke, 1976). The reaction mixture contained 50 mM potassium phosphate buffer (pH 8.3), 1 mM EDTA, 1mM sodium azide, 0.2 mM NADPH, and 1 U/ml glutathione reductase. The reaction was initiated with the addition of 1.5 m Mcumenehydroperoxide. The enzyme activity was estimated from the rate of oxidation of NADPH (E340=6200$M^{-1}cm^{-1}$) and the enzyme activity was expressed as mmol/minute/ milligram protein.

Lipid peroxidation (LPO) concentration

For the evaluation of LP concentration we measured malondialdehyde (MDA) by the TBARs method at 535 nm (Buege and Aust, 1978). 2 ml of the reaction mixture [thiobarbituric acid (0.375%), trichloroacetic acid (15%) and hydrochloric acid (0.25 N)] were mixed in 1:1:1 and were added to 1 ml of the heat denatured supernatant. TBARs levels were estimated at 535 nm using MDA as standard. Total protein concentration was measured on the supernatants (10,000g during 20 min at 4^0C) of the homogenized tissues (Stoscheck, 1990). The concentration of LP compounds was expressed as n moles of MDA formed per mg protein.

Statistical analysis

Data were analyzed through a one-way analysis of variance (ANOVA) followed by post hoc test, Tukey's

multiple range test ($P < 0.05$). Data were expressed as mean ± standard error mean (SEM).

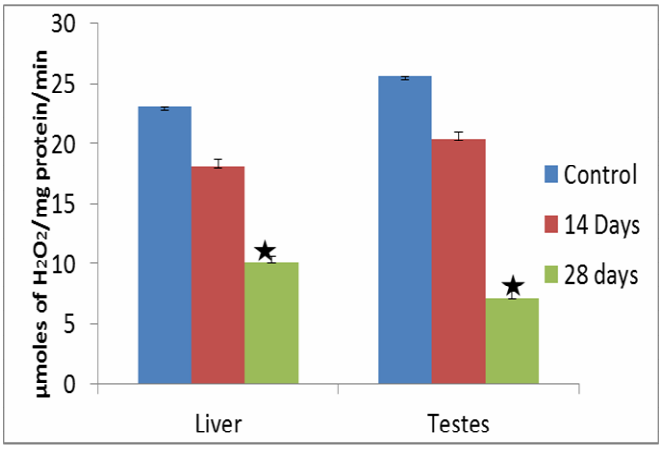

Fig. 1 : Catalase (CAT) level in the liver and testes of a freshwater catfish, *H. fossilis* exposed to PCP for 14 and 28 days. (Asterisks shows significant difference from Control at P<0.05; ANOVA following Tukey's post hoc Test)

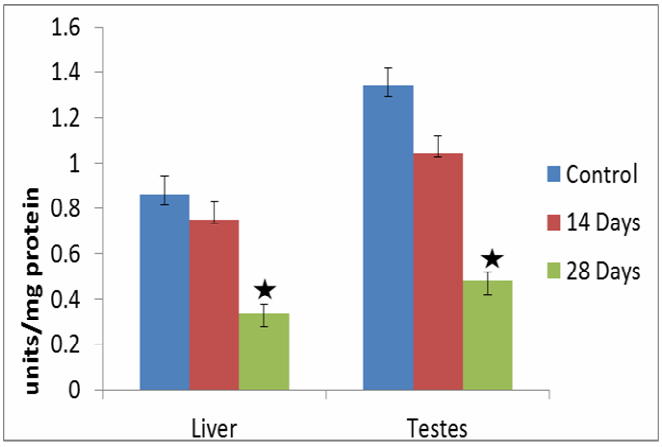

Fig. 2 : Super Oxide Dismutase (SOD) level in the liver and testes of a freshwater catfish, *H. fossilis* exposed to PCP for 14 and 28 days. (Asterisks shows significant difference from Control at P<0.05; ANOVA following Tukey's post hoc Test)

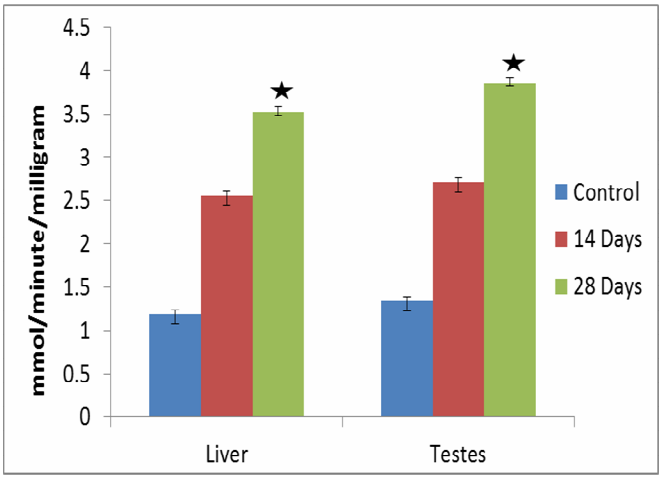

Fig. 3 : Glutathione Peroxidase (GPx) level in the liver and testes of a freshwater catfish, *H. fossilis* exposed to PCP for 14 and 28 days. (Asterisks shows significant difference from Control at P<0.05; ANOVA following Tukey's post hoc Test)

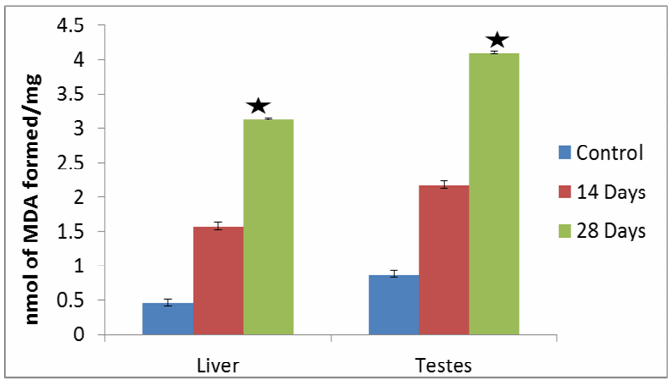

Fig. 4 : Lipid Peroxidase (LPO) level in the liver and testes of a freshwater catfish, *H. fossilis* exposed to PCP for 14 and 28 days. (Asterisks shows significant difference from Control at P<0.05; ANOVA following Tukey's post hoc Test)

Results

SOD (0.48; 0.34 units/milligram protein) activity was found to be decreased as compared to control groups (1.34; 0.86 units/milligram protein) and CAT activity was also observed to be lower in testes and liver (7.12; 10.07 micromoles of H_2O_2 metabolized/milligram protein/minute) than in the control groups (25.61; 23.08 micromoles of H_2O_2 metabolized/milligram protein/minute) respectively.

The findings showed elevated level of MDA in testicular (4.09 nmoles of MDA per gram of wet mass) and hepatic tissue (3.13 nmoles of MDA per gram of wet mass) than in control testes (0.86 nmoles of MDA per gram of wet mass) and liver (0.45 nmoles of MDA per gram of wet mass) of fish signifying free radical induced cell injury due to oxidative stress. GPx was also found to be significantly high in testicular (3.86 mmol/minute/milligram protein) and hepatic tissue (3.52 mmol/minute/milligram protein) than in control testes (1.34 mmol/minute/milligram protein) and liver (1.19 mmol/minute/milligram protein) respectively.

Discussion

Various industries (pulp, rubber, glass, sugar, tannery, steel plants, chemicals, plastics etc.) alongside the banks of river discharge the waste directly into water body. Studies on pollutant-induced toxicity in fish have revealed bioaccumulation of heavy metals in different tissues (Sen *et al.*, 2011; Fatima and Usmani, 2013) thereby, affecting the cellular architecture and physiology. Hence, study of fish as affected animal provides an insight into the potential toxic effects of perilous aquatic environments (Tyor and Pahwa, 2017). The exposure to environmental contaminants may produce oxidative damage by generating free radicals and/or altering antioxidant enzyme systems (Huang *et al.*, 2007). Free radical (molecule with one or more unpaired electrons in a single orbit) produced as a result of ATP production by mitochondria become a part of the propagative chain reaction whereby they combine with other radicals to form other more damaging species, unless the chain is terminated by antioxidants to form a nontoxic species (Halliwell, 1994). The free radical immediately react with lipids in the membranes of cell and organelles, proteins and DNA eventually forming damaging products such as lipid peroxides and other lipid adducts. The indicator of lipid peroxidation includes measurement of TBARS content (Wheatley, 2000) which provides relative measure of the potential for pollutants to cause oxidative injury

(Vlahogianni *et al.*, 2007). During normal physiological status anti-oxidant defense systems provides compensatory response to oxidative damage by removing reactive oxygen species (Livingstoone, 2001) and thus, have been considered as biomarkers of contaminant, its induction reflects a protective response to pollutants (Borkovic *et al.*, 2005). An increase in the LPO level was observed after fluoride exposure in the liver and ovary of fishes (Basha and Sujitha, 2012). Increase in MDA was an indicator of enhanced oxidative stress (Buyukokuroglu *et al.*, 2002). Significant increase in MDA content was observed in Dichlorvos exposed *H. fossilis* (Vadhva and Hasan, 1986). Increased MDA level was also observed in the study carried out by scientist (Hai *et al.*, 1997) in *Cyprinus carpio* and *Ictalurus nebulosus* following the exposures to dichlorvos. SOD has been known as the first defense line of cell against ROS and could protect against superoxide induced oxidative damage (Fridovich, 1989) by catalyzing the conversion of the superoxide anion radical to molecular oxygen and hydrogen-peroxide (H_2O_2) while, CAT is involved in mitigating the toxic effects of H_2O_2 by decomposing it into water and oxygen. Inhibition of catalase activity may be due to overproduction of superoxide anion radical (Pandey *et al.*, 2003) or due to presence of nitrites (Arrillo and Melodia, 1991).

In the present investigation tissue specific responses in the activities of biomarker antioxidant enzymes such as SOD which detoxifies toxic superoxide anion radicals, catalase known to be primary antioxidant defense component, GPx and LPO were observed for assessing the PCP toxicity.

The findings of present study revealed significant decrease in the activities of SOD, CAT enzymes in liver and testes of fish when compared to control. Thus, the decrease in both the enzymes may be suggestive of the reason that their activity might be inhibited by the pollutants. The breakdown of H_2O_2 to water and oxygen is done by CAT, thereby, protecting the cell from the damaging action of H_2O_2 and the hydroxyl radical. SOD and CAT level decreased significantly in testes and liver when exposed to 32µg/l concentration of PCP. The PCP exposure increased the GPx and LPO activity in testes and liver of fish compared to control group. The elevation of antioxidant activities in organism might be indicative of the adaptive response of organisms to counteract the oxidative effect of reactive oxygen species generated by PCP exposure.

Reports discussed that GPx catalyzes the reaction of hydro peroxides with reduced GSH to form glutathione disulfide and the reduction product of the hydro peroxide and they further added that the decrease in activity of amino-transferases in the liver of fish *Oreochromis mossambicus* exposed to organo-phosphorus pesticide may be due to liver damage (Rao, 2006). In a recent study the decreased GOT and GPT in gill, liver and muscle of fish *Cyprinus carpio* after carbamazepine exposure suggested that detoxification mechanism may not be sufficiently effective to prevent the animal from toxicity and effect of drug on the system (Malarvizhi *et al.*, 2012). Furthermore, in some research it is suggested that the decrease in the activities of the metabolic enzymes during organophosphorus pesticides exposure may be due to the direct action of these pesticides on enzymes (Tripathi and Shasmal, 2011). Both mammalian and piscine systems shows general mechanisms of oxidative toxicity which appear more or less similar with comparable lesions and its responses, serve as markers of oxidative stress (Vutukuru *et al.*, 2006). The oxidative stress caused by pesticides in aquatic organisms may lead to ROS production and alterations in antioxidants enzymes (Livingstone, 2001). The excessive production of ROS production and their damaging effects can be minimized by the cellular antioxidant systems (Sureda *et al.*, 2004; Dorts *et al.*, 2012). Scientists also advocated that organisms under stress conditions utilizes the antioxidant enzymes to adapt to environmental stress and altered activities depend on the dose, species and route of exposure (Gravato *et al.*, 2006). The enzymes such as catalase and SOD play a major role in counteracting the ROS produced during bioactivation of Xenobiotics and the induction of CAT/SOD system might be the foremost defense mechanism against ROS (Nwani *et al.*, 2010). Moreover, it is stated in some reports that SOD and CAT are highly sensitive enzymes respond quickly to protect organisms from prevailing oxidative stress (Rao *et al.*, 2006; Dewez *et al.*, 2005). SOD is the first enzyme that responds to oxidative stress during any stress condition in animals (McCord and Fridovich, 1969; Winston and Di Giulio, 1991). The elevation of antioxidant activities in organism generally indicates adaptive responses of organisms to counteract the oxidative effect of generated reactive oxygen species (Hegazi *et al.*, 2010).

Decrease in catalase activity observed in the liver and testes of fish in exposed group may be due to its adaptive response to H_2O_2 produced by SOD activity, since, catalase is responsible for its detoxification to water (Nwani *et al.*, 2010; Elia *et al.*, 2002). The variation in the activity of antioxidant enzymes may as indicators of pollutant mediated oxidative stress (Sayeed *et al.*, 2003). MDA is the final product of lipid peroxidation and their concentration in animals can be taken as direct evidence of toxic process caused by free radicals (Sieja and Talerczyk, 2004). The elevation of LPO content in and liver indicates an increase in free radicals due to toxicity or metabolism (Freeman and Crapo, 1981; Gultekin and Akdogan, 2000; Ruas *et al.*, 2008). The results of the present investigation indicate that exposure of PCP induces significant changes in the enzymatic profiles in *H. fossilis.* The PCP exposure to the fish indicated the oxidative stress in the body by generating imbalance between reactive oxygen species (ROS) and antioxidants. The presence of such level of PCP in the natural environment is dangerous to the ecosystem and will definitely affect the survival of fish. We are further interested in studying the assessment of residual amount of this pesticide in different body tissues of fish for further understanding.

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