Abstract

Study on the chronic effects of an organophosphate, chlorpyrifos inducing oxidative stress in freshwater culturable carp, *Ctenopharyngodon idellus* has been made on exposure to sub-lethal concentration (1.41µg/L and 2.44µg/L) of the pesticide for 15, 30 and 60 days. Antioxidants viz. catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), glutathione-s-transferase (GST) and lipid peroxidation (LPO) in the liver, kidney and gills of the fish were analyzed. Studies revealed that the enhanced productions of reactive oxygen species (ROS) lead to oxidative damage to lipids & proteins, and inhibit antioxidant defence system of fish. The activity of LPO has followed an increasing trend and a decline in the activity of CAT, SOD and GSH at both the concentrations throughout the experiment. However, no definite trend in the activity of GST has been observed. Prolonged exposure to chlorpyrifos enhanced ROS formation, finally resulted in oxidative damage to cell and inhibited antioxidant capacities in the fish tissues.

Keywords: Oxidative stress; Chlorpyrifos; *Ctenopharyngodon idellus*; Toxicity

Abbreviations: CAT: Catalase; CPF: Chlorpyrifos; EC: Emulsifying Concentrate, GPx: Glutathione Peroxidase; GSH: Reduced Glutathione; GSSG: Oxidised Glutathione; GST: Glutathione -s-Transferase; LC50: Lethal Concentration at Which 50% Mortality Occurs; LPO: Lipid Peroxidation; MDA: Malondialdehyde; OP: Organophosphates; PCA: Principal Component Analysis; PMS: Post Mitochondrial Solution; ROS: Reactive Oxygen Species; SOD: Superoxide Dismutase; TBARS: Thiobarbituric Acid Reactive Substances

Introduction

Even though the use of pesticides had a very positive impact in the overall increase in food production, the risks associated with this include deterioration of human health, water contamination, animal poisoning, and death of beneficial insects, wildlife endangerment and pesticide tolerance [1]. But at the same time, these are responsible for pollution of our aquatic resources. They carried into water bodies like ponds and rivers through surface runoff and alter the physico-chemical properties of water. In India, the pesticide consumption has increased by more than fourfold after Green Revolution era (1966-1999) and the agricultural products have been found to contain substantial quantities of pesticide residues. The major reason is indiscriminate use of chemical pesticides. Pesticides are included in a broad range of organic micro-pollutants that have tremendous ecological impacts. Different categories of pesticides have different effects on living organisms and hence generalization is difficult. Although terrestrial impacts by pesticides do occur, the principal pathway that causes ecological impacts is that of water contaminated by pesticide runoff. Among pesticides, organophosphates (OP) are used most frequently used throughout the world, because of their relatively lesser persistence in the soil.

Among the various organisms present in the aquatic ecosystem, fishes are the one that are relatively more sensitive to changes in their surrounding environment. The pesticide concentration in the aquatic organisms appears to be several times higher than the concentration present in the ecosystem. This is due to bioaccumulation in which toxic substances are taken up from the environment by the organism and get accumulated in various organs and tissues. Toxic substances also become increasingly concentrated at higher trophic levels possibly due to biomagnifications [2]. The pesticides which are liberated into aquatic ecosystem have a tremendous effect on fish, and thereby to man [3].

With many organophosphorus insecticides, an irreversibly inhibited enzyme is formed and the signs and symptoms of intoxication are prolonged and persistent. However, some organ phosphorus insecticides are thought to be toxic only after metabolism by enzyme systems and they could trigger enzymological variations in fishes. Chlorpyrifos is a broad-spectrum, organophosphate with wide application in the field of agriculture and has wide variation of toxicity among different species. It is the second largest selling OP agrochemical in India [4].

Pesticide residues in the aquatic environment, poses toxicological hazards to a myriad of non target organisms [5], and finally finding their way to the food chain. Pollutants have high potential to induce oxidative stress in aquatic organisms through production of free radicals and reactive oxygen species...
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(ROS) and induce an imbalance between intracellular ROS levels and antioxidant protection, and can subsequently cause oxidative stress in organisms [6,7]. ROS generated can cause damage to proteins, lipids, carbohydrates and nucleic acids [8]. The resulting damage may alter cell functions, eventually leading to cell death [9,10]. The antioxidants in fish could be used as biomarkers of exposure to aquatic pollutants. Keeping in view, present work has been undertaken to evaluate the oxidative stress parameters of liver, kidney and gills of Ctenopharyngodon idellus, exposed to the different sub-lethal concentrations of chlorpyrifos.

Materials and Methods

Animal collection and acclimatization

Fingerlings of Ctenopharyngodon idellus (wt. 10±2 gm, length, 10±2 cm), was collected from Nanoke Fish Seed Farm located at Nanoke village, District Patiala, (Punjab, India) and were safely brought in oxygen water packed polythene bags. They were acclimatized to the laboratory conditions for 15 days in glass aquarium and fed with pelletized supplementary feed once a day at least 1 h prior to replacement of water. The physico-chemical characteristics of water used in the experiment were determined in accordance with the standard methods [11] (pH 7.2±0.1, temperature 25±2 °C, dissolved oxygen 8.0±0.3 mg/L, total alkalinity 175±10 mg/L and total hardness 18±0.5 mg/L).

Chemicals

Chlorpyrifos (20% EC), commercial grade was purchased from Shivalk Insecticide Pvt. Ltd., India. Stock solution was prepared by dissolving the appropriate amount of chlorpyrifos in distilled water weekly and was further used for making concentration for acute toxicity tests. Other chemicals of analytical grade (CDH, New Delhi) were purchased from local scientific suppliers, Chandigarh, India.

Experimental design

To study the acute toxicity test of chlorpyrifos, the experimental fish were exposed to different concentrations of chlorpyrifos (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5 µg/L) for 96 h. On the basis of fish mortality response and chlorpyrifos concentration, the LC50 of chlorpyrifos by Probit analysis [12] was found to be 7.24 µg/L for 96 h. For chronic studies, fish were exposed to 1/3 of LC50 (2.41 µg/L) and 1/5 of LC50 (1.44 µg/L) for 15, 30 and 60 days. A control set of fish was used with each experimental group (20 fish in each group). Experiments were conducted in duplicates along with the control. During the experiment the water was changed daily to avoid the accumulation of fecal matter and to maintain the toxicant concentration. Biochemical analysis of liver, kidney and gills of the fish exposed to the toxicant and of control was made on 15th, 30th and 60th day. For this, at the end of each exposure, the fish were sacrificed by cervical dislocation. The organs were excised and immediately washed in physiological saline solution.

Biochemical Analysis

Tissue preparation

Each organ was homogenized (10% w/v) in 0.1 M Tris HCl buffer (pH 7.4) using Porter-Elvehjem homogenizer at 0-4°C. The homogenate was centrifuged at 9200 rpm for 10 min to extract post mitochondrial supernatant (PMS), stored at -20 °C and analysed to estimate superoxide dismutase (SOD), catalase (CAT) and glutathione-s-transferase (GST). Lipid peroxidation (LPO) and reduced glutathione (GSH) were estimated from homogenate by measuring optical density on Perkin Elmer Lamda 35 uv/vis spectrophotometer. All the measurements were made in duplicate.

Lipid peroxidation

LPO was estimated by a TBARS (thiobarbituric acid-reactive substances) assay, performed by malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA) [13]. Homogenate (0.1 mL) was added in 0.1mL each of 150 mM Tris-HCl (pH-7.1), 1.5 mM ascorbic acid and 1.0 mM ferrous sulphate in a final volume of 1 mL and incubated at 37°C for 15 min. To this, 1 mL of 10% trichloroacetic acid (TCA) and 2 mL of 0.375% thiobarbituric acid were added & kept in boiling water bath for 15 min. The contents were centrifuged at 3000 rpm for 10 min and optical density was measured by at 532 nm.

Superoxide dismutase

The reaction mixture containing 1.2 mL of solution A (50 mM sodium carbonate in 0.1 mM EDTA buffer, pH 10.8), 0.5 mL solution B (96 µM NBT) and 0.1 mL of solution C (0.6% Triton X-100) were incubated at 37°C for 10 min. Reaction was initiated by adding 0.1 mL of 20 mM hydroxylamine HCl (pH 6). The rate of NBT dye reduction by O-2 anion generated due to photoactivation of hydroxylamine HCl was recorded at 560 nm for 3 min for blank. Then 0.1 mL PMS was immediately added after addition of hydroxylamine HCl to the reaction mixture. After mixing thoroughly, 50% inhibition in the rate of NBT dye reduction by SOD present in the enzyme source was recorded at 560 nm for 3 min [14].

Catalase

The assay mixture was prepared of 2.9 mL of 125 mM H2O2 & 0.067 M phosphate buffer (pH 7.0) and 0.01 mL PMS (DDW for blank). The decrease in absorbance/30 sec at 240 nm was measured for 3 min [15].

Reduced glutathione

mL of homogenate and 2.0 mL of 0.2 M phosphate buffer were mixed thoroughly followed by addition of 1 mL of 1.0 mM dithio-bisnitro benzoic acid (DTNB) prepared in 1% (w/v) potassium citrate. The contents were centrifuged at 3000 rpm for 15 min and absorbance of the supernatant was measured against reference blank at 412 nm [16].

Glutathione S-transferase: PMs was incubated in 1 mL of 0.2 M phosphate buffer (pH-6.5), 0.1 mL of 20 mM 1-chloro-2, 4-dinitrobenzene (CDNB) prepared in 95% ethanol and 0.8 ml of DDW. After mixing thoroughly, incubation was carried out at 37°C for 5 min. To this, 0.1 mL of 20 mM GSH (dissolved in DDW) was added just before measuring the increase in absorbance/30 sec at 340 nm for 5 minutes. In case of blank, 2.9 ml phosphate buffer and 0.1 mL CDNB were mixed [17].
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Total Proteins

Protein was determined in homogenate and PMS by the Lowry’s reagent using bovine serum albumin as standard and absorbance of the sample was measured at 595 nm [18].

Statistical Analysis

Statistical analysis was carried out using one-way ANOVA with Tukey’s test. The data were subjected to a principal component analysis (PCA). Statistical relationships between oxidative stress variables in gills, liver and kidney of grass carp were also compared using non-parametric Spearman’s rank correlation.

Results and Discussion

LC50

The calculated 96 h LC50 value (95% confidence limits) of chlorpyrifos using a static bioassay to fingerlings Ctenopharyngodon idellus was 7.24 µg/L [19].

Biochemical analysis

The most intensively studied biochemical biomarkers are the enzymes involved in the process of detoxification of xenobiotics and their metabolites. Fish have biotransformation enzymes, and their main function is to catalyze the conversion of liposoluble compounds to more excretable metabolites [20]. This process of biotransformation involves Phase I detoxification process by hydrolyzing the toxic compounds, which may be excreted out or continued on biotransformation pathway [21]. In Phase II detoxification process, there occurs the conjugation of the metabolites produced in Phase I with the cellular endogenous compounds [22]. During the transformation of toxic agent, reactive oxygen species are formed that can damage cell structures via oxidation. Oxidative stress is the imbalance between the production of free radicals capable of causing peroxidation of lipid membranes of the cells and the antioxidant defense system of the body. Free radicals formation occurs in the cell continuously as a result of both enzymatic and non-enzymatic reactions and causes oxidation of proteins, DNA and peroxidation of unsaturated lipids in the cell membranes. This produces highly unstable lipid hydroperoxides, whose products are highly reactive on decomposition; as a result the products can break down into free radicals and threaten cell integrity [23]. Imbalance in the cellular redox system may be used as markers of oxidative stress [24].

Lipid Peroxidation (LPO)

Pesticides may induce oxidative stress leading to the generation of free radicals and cause lipid peroxidation as molecular mechanism [25]. LPO is particularly important for aquatic animals since they normally contain greater amount of polyunsaturated fatty acids than others, which has been reported to be a major contributor to the loss of cell function [26]. It is the initial step of cellular membrane damage caused by pro-oxidants and xenobiotics [21]. It is considered as an indicator of oxidative damage of cellular components [27] and is analyzed in terms of thiobarbituric acid reactive substances (TBARS). In liver of the fish exposed to chlorpyrifos for 15 days, significant (p< 0.01) increase (3.4%) in LPO level as compared to control, at lower concentration of the toxicant has been observed, though the elevation (5.5%) observed at higher concentration has been found to be insignificant. On contrary, after 30 days exposure the elevation in LPO level (31%) at higher concentration has been found to be significant (p< 0.05), though the change (16.5%) at lower concentration of the toxicant has been found to be insignificant. On 60th day, a significant (p< 0.05) increase in LPO level (40.2%) at lower concentration has been found, and at higher concentration insignificant change (59.1%) occurred (Table 1).

Table 1: Variation in LPO level (µmoles/MDA/mg protein) in liver, kidney and gills of C. idellus on exposure to chlorpyrifos.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control 1.44 µg/L</th>
<th>2.41 µg/L</th>
<th>30 days</th>
<th>Control 1.44 µg/L</th>
<th>2.41 µg/L</th>
<th>60 days</th>
<th>Control 1.44 µg/L</th>
<th>2.41 µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.146±</td>
<td>0.151±</td>
<td>0.155±</td>
<td>0.145±</td>
<td>0.169±</td>
<td>0.19±</td>
<td>0.144±</td>
<td>0.202±</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>0.001*#</td>
<td>0.0015</td>
<td>0.001</td>
<td>0.005</td>
<td>0.005*#</td>
<td>0.0008</td>
<td>0.004*</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.134±</td>
<td>0.135±</td>
<td>0.138±</td>
<td>0.132±</td>
<td>0.140±</td>
<td>0.144±</td>
<td>0.133±</td>
<td>0.151±</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>0.0005*#</td>
<td>0.001</td>
<td>0.002</td>
<td>0.0005*</td>
<td>0.001</td>
<td>0.003</td>
<td>0.02*</td>
</tr>
<tr>
<td>Gills</td>
<td>0.125±</td>
<td>0.127±</td>
<td>0.129±</td>
<td>0.124±</td>
<td>0.130±</td>
<td>0.136±</td>
<td>0.123±</td>
<td>0.154±</td>
</tr>
<tr>
<td></td>
<td>0.0005</td>
<td>0.0005*#</td>
<td>0.0005**</td>
<td>0.001</td>
<td>0.005*</td>
<td>0.001</td>
<td>0.001</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Data is presented as Mean ± S.D., n= 6

*p< 0.05, significant difference: control vs. 1.44 µg/L and 2.41 µg/L CPF treated group.

#p< 0.01, significant difference: control vs. 1.44 µg/L and 2.41 µg/L CPF treated group.

In kidney of the fish exposed to CPF for 15 days, LPO increased (0.74%) significantly (p< 0.01) at 1.44 µg/L of CPF as compared to control, and at 2.41 µg/L/ no significant change (2.9%) has been found. On 30 days exposure, similar significant (p< 0.05) increase (6.05%) in LPO has been noticed at lower concentration and the change (9.09%) has been found to be insignificant at higher concentration of the toxicant. Whereas after 60 days exposure, significant (p< 0.05) increase (13.53%) and 17.2% has been observed at both the toxicant concentrations (Table 1).

In gills on exposure of the fish to the toxicant, for 15 days, significant (p < 0.01) increase (1.6% and 3.2%) in LPO level has been found at both lower and higher concentration of the toxicant respectively. On 30th day exposure, significant (p < 0.05) increase in LPO level (9.6%) has been observed at higher concentration and at lower concentration respectively. After 60 days exposure of the fish to CPF, an insignificant change (25.02% and 35.77%) has been found at both the concentrations of the toxicant (Table 1). Enhanced level of LPO has been observed in liver, kidney and gills of the fish exposed to both sublethal concentrations of chlorpyrifos at all the exposure periods. Induced level of LPO has also been reported [28-30]. This, as explained [31], could be attributed to generation of a high level of free radicals which lead to destabilization and disintegration of cell membrane, thereby damage the organ. This is in agreement with the findings of other workers on different fishes (Oncorhynhus mykiss exposed to propiconazole [32], Danio rerio exposed to atrazine [33] Carassius auratus exposed to sencor [34]. Carassius carassius exposed to endosulphan [5]. The increase in TBARS level could be the result of impairment in antioxidant enzymes due to enhanced ROS formation, resulted in cell membrane damage and cellular dysfunction. This is further evidenced by ultrastructural alterations like loss of functional unit of mitochondria, peroxisomes and endoplasmic reticulum in liver, kidney and gills of the fish [6,35]. As reported in one of our work [36], other workers [37] also reported that the cholinergic hypersensitivity induced by the inhibition of AChE initiates the accumulation of ROS, leading to lipid peroxidation, on exposure of the fish to organophosphate compounds has also bee

**Superoxide dismutase (SOD)**

SOD is a group of metalloenzymes that plays a crucial antioxidant role and constitutes the primary defense against the toxic effects of superoxide radicals in aerobic organisms. SOD catalyses the transformation of superoxide radicals to H2O2 and O2 and it is first enzyme to cope with oxi-radicals [38]. In liver, SOD activity increased (1.28%) significantly (p < 0.05) at 1.44 µg/L as compared to control, though the change (2.9%) has not been found to be significant at higher concentration of the toxicant. Contrary to this, on 30th day, significant (p < 0.05) decrease (0.47%) in SOD activity has been found at 1.44 µg/L of CPF as compared to control, though the change (2.9%) has not been found to be significant at higher concentration of the toxicant. Contrary to this, on 30th day, significant (p < 0.05) decrease (0.47%) in SOD activity has been found at 1.44 µg/L of CPF and the change (2.23%) was not significant at 2.41 µg/L of CPF. After 60 days exposure, a significant (p < 0.05) reduction (0.96% and 2.56%) from control has been observed at lower and higher sublethal concentration of CPF respectively (Table 2). SOD activity in kidney of C. idellus after exposure to CPF showed significant (p < 0.05) increase (2.01%) at higher concentration on 15th day, whereas at lower concentration no significant change (1.20%) has been observed. Further, with increased exposure, SOD activity showed declining trend. Significant (p < 0.01) decrease (4%) occurred on 30th day exposure at 1.44 µg/L of CPF and (8.81%) on 60 day exposure at 2.41 µg/L CPF. The change (7.6%) observed has not been found to be significant at higher concentration on 30th day and (2.82%) at lower concentration on 60th day (Table 2). A fluctuating trend has been observed in SOD activity in gills of the fish exposed to chlorpyrifos. On 15th day exposure, SOD activity increased (3.33%) significantly (p < 0.05) at higher concentration, and at lower concentration, much change (1.11%) did not occur. On 30th day exposure, significant (p < 0.01) decrease in SOD activity (24.8%) occurred at higher concentration of CPF and insignificant decrease (19.10%) at lower concentration of the toxicant, whereas the decrease (10.71% and 13.2%) observed at both the concentrations on 60th day exposure has been found to be insignificant (Table 2). The initial increase of SOD activity observed during present investigation in the tissues of the fish demonstrates that chlorpyrifos induced adaptive response and it is scavenging the overproduction of superoxide ions under the oxidative stress. Therefore, an increase in SOD activity indicates that there is O2-generation and this generation can still be eliminated. Otherwise, with the overproduction of superoxide anions to an extent exceeds the function of SOD elimination, thus these anions can inactivate the enzyme. Variation in SOD activity has also been reported by other workers [39,35]. Later, with increasing exposure period and toxicant concentration, the SOD activity got decreased, and this might attributed to dismutate O-2 and to decompose hydrogen peroxide. The antioxidant enzymes fluctuate by toxicants; interact primarily with the tissues, resulting in fluctuated enzyme activity by increased production of reactive oxygen species as a result of oxidative stress. This, as pointed out [40], the superoxide anions cause oxidation of cytochrome in enzyme by themselves or after their transformation to hydrogen peroxide. The altered SOD activities might have reflected a cellular oxidative stress due to exposure to the toxicant. Similar findings have been made on the effect of pesticides on other fishes (Carassius auratus exposed to round up [41], Oreochromis niloticus exposed to chlorpyrifos [39], Cyprinus carpio exposed to terbutryrin [42]. Carassius carassius exposed to endosulphan [5].

**Table 2: Variation in activity of SOD (units/min/mg protein) in liver, kidney and gills of C. idellus on exposure to chlorpyrifos.**

<table>
<thead>
<tr>
<th>Organ</th>
<th>15 days</th>
<th>30 days</th>
<th>60 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>1.44 µg/L</td>
<td>2.41 µg/L</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.48±</td>
<td>2.45±</td>
<td>2.53±</td>
</tr>
<tr>
<td></td>
<td>0.015</td>
<td>0.01</td>
<td>0.02*</td>
</tr>
<tr>
<td>Gills</td>
<td>0.90±</td>
<td>0.94±</td>
<td>0.98±</td>
</tr>
<tr>
<td></td>
<td>0.015</td>
<td>0.02</td>
<td>0.01*</td>
</tr>
</tbody>
</table>

Data is presented as Mean ± S.D., n = 6.

*p < 0.05, significant difference: control vs. 1.44 µg/L and 2.41 µg/L CPF treated group.

#p < 0.01, significant difference: control vs. 1.44 µg/L and 2.41 µg/L CPF treated group.

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Further, [28] have suggested that the activity of antioxidant may be increased or decreased under chemical stress depending on the intensity and the duration of stress applied as well as susceptibility of exposed species and they explained that this could be due to overproduction of superoxide radicals. From the present findings, it could be inferred that different tissue responses have been observed in liver, kidney and gills of the *C. idellus* exposed to chlorpyrifos. The initial increase in SOD activity might be due to increased generation and overproduction of reactive oxygen species, while decreased SOD activity was due to direct damage of its protein structure by pesticide and enhanced amount of hydrogen peroxide.

### Catalase (CAT)

Catalase is an enzyme that is located in the peroxisomes and facilitates the removal of hydrogen peroxide, which is metabolized to oxygen and water [43]. The activity of CAT in liver showed fluctuating trend throughout the exposure. On 15th day, CAT activity increased (0.71%) significantly (p = 0.01) at lower concentration of the toxicant, though at higher concentration the elevated activity (1.6%) has been found to be insignificant. On 30th day exposure, significant (p = 0.05) decrease in CAT activity (0.178%) at lower concentration has been noticed, whereas at higher concentration significant (p < 0.05) increase (0.4%) occurred. However, significant (p < 0.05) marked decline in CAT activity (1.78% and 2.4%) has been noticed on 60th day exposure at both the concentrations of the toxicant (Table 3). In kidney of the fish, activity of CAT showed significant (p < 0.05) increase (2.57%) from control after 15 days exposure at 2.41 µg/l CPF, and at 1.44 µg/l insignificant increase (1.15%) occurred. On 30th day, CAT activity showed similar significant (p < 0.05) increase (0.89%) at higher concentration of the toxicant, and at lower concentration no significant change (0.15%) has been observed. After 60 days, similar significant (p < 0.05) increase in CAT activity (0.36%) has been observed at higher concentration, whereas at lower concentration an insignificant decline in CAT activity (0.10%) has been noticed (Table 3). All the significant changes in CAT activity in kidney of the fish have been found at higher concentration of the toxicant. In gills of the fish exposed to CPF, CAT activity showed significant (p < 0.05) decrease (4.75%) from control at higher concentration of the toxicant after 15 days exposure, whereas at lower concentration of the toxicant the decreased CAT activity (2.34%) has been found to be insignificant. On contrary, after 30 and 60 days exposure significant (p < 0.05) decrease in CAT activity (4.9% and 7.2%) has been observed at lower concentration of the toxicant, though the change has been found to be insignificant (8.54% and 10%) at higher concentration of the toxicant (Table 3).

**Table 3: Variation in activity of SOD (units/min/mg protein) in liver, kidney and gills of *C. idellus* on exposure to chlorpyrifos**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Control</th>
<th>15 days</th>
<th>30 days</th>
<th>60 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>1.14 µg/L</td>
<td>2.41 µg/L</td>
<td>1.14 µg/L</td>
<td>2.41 µg/L</td>
</tr>
<tr>
<td>Kidney</td>
<td>19.04±</td>
<td>19.26±</td>
<td>19.53±</td>
<td>19.07±</td>
</tr>
<tr>
<td>Gills</td>
<td>6.64±</td>
<td>6.49±</td>
<td>6.33±</td>
<td>6.60±</td>
</tr>
</tbody>
</table>

Data is presented as Mean ± S.D, n = 6.

* p < 0.05, significant difference: control vs. 1.44 µg/L and 2.41 µg/L CPF treated group.

# p < 0.01, significant difference: control vs. 1.44 µg/L and 2.41 µg/L CPF treated group.

During present investigation, CAT activity in gills found to be decreased at all the three exposures both at low and high toxicant concentrations. This could be explained due to the influx of superoxide radicals inhibiting the CAT activity and inability to set up adequate compensation in presence of toxicant [44,29]. Further, respiratory system provides the most extensive interface of fish with the aquatic environment, being frequently the first system affected by toxicants [45,46]. Decrease in CAT activity in gills was also reported in other fishes (*Oreochromis niloticus* & *Cyprinus carpio* exposed to 2,4-D & azinophomethyl [47], *Cyprinus carpio* exposed to diazinon [28], *Oncorhynchus mykiss* exposed to propiconazole [32]). *Cyprinus carpio* exposed to prometryne [35]. However, some authors [48] could not find any significant change in CAT activity. This could be due to very short exposure of the toxicant. Other workers [28] found slight increase in CAT activity on 5th day exposure. The increase in CAT activity in chlorpyrifos exposed kidney of *C. idellus* observed during present investigation could be explained as an adaptive response of the fish. Further, the higher CAT activity might be in response to increased oxygen consumption giving a great potential for hydrogen peroxide production [49]. Similar findings have been made [28] on *Oreochromis niloticus* & *Cyprinus carpio* exposed to 2,4-D & azinophomethyl. However, some authors [33,34] have reported a decrease in CAT activity in the kidney of the fish. This might be due to quite higher concentration of the pesticide used. CAT activity observed in the liver of the treated fish showed initial increase & followed by decrease in the subsequent exposure. The initial elevation in the enzyme activity indicates an elevated antioxidant level in order to neutralize the impact of ROS. Further, the decrease in CAT activity could be due to influx...
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of superoxide radicals. Present observations are in concurrence with the findings of other workers [jennyis multidentata exposed to endosuphan [48], Rhamdia quelen exposed to agrochemicals [31] Cyprinus carpio exposed to tebucinazo [6], Cyprinus carpio exposed to prometryne [35]. Initial increase due to the effect of methyl parathion on Brycon cephalus [45] and atrazine on Danio rerio [50] has also been reported. Fluctuation in CAT activity due to the effect of the toxicant exposure as superoxide radicals and hydrogen peroxide may lead to hydroxyl radical formation again and enhanced hydroxyl radicals formation or weakened cellular antioxidant defense can stimulate free radical chain reaction by interacting with proteins, lipids and nucleic acids causing cellular damage [51]. Present findings indicate that gills are the most sensitive organ to oxidative stress in comparison to liver and kidney. These differences could be because of different rates of free radicals generation & different antioxidant potential in the tissues. The detoxification system of the gills is not as robust as that of liver and kidney, which increases its vulnerability towards ROS [52].

Reduced glutathione (GSH)

GSH is a major cytosolic low molecular weight sulhydryl compound that acts as cellular reducing and protective reagent against a wide range of pollutants through SH-group [53]. It directly acts a scavenger of oxyradical and also as an antioxidant enzyme substrate [46]. Apparently GSH is important in protecting against deleterious effects of the cell exposed to ROS by reacting with them to form glutathione disulphide (GSSG). This antioxidant defense effect occurs spontaneously through GSH or by GST [54]. It acts as cofactor for glutathione transferase, which facilitates the removal of certain chemicals and other reactive molecules from the cells [55]. Thus a change in GSH levels may be an important indicator of detoxification ability of an organism [56]. The level of GSH in liver, kidney and gills followed declining trend throughout the exposure periods. In liver, GSH level decreased (4.65% and 8.67%) significantly (p<0.01) from control at lower and higher concentration of the toxicant respectively after 15 days exposure, whereas on 30th day, significant (p< 0.05) decrease in GSH level (11.48%) has been noticed at higher concentration and at lower concentration the decrease (10.56%) has been found to be insignificant. Similar significant (p< 0.05) marked decrease by 1.14 folds (12.3%) in GSH level at higher concentration of the toxicant has been noticed, though the change (11.41%) has not been found to be significant at lower concentration of the toxicant (Table 4).

Table 4: Variation in GSH (µmoles/min/mg protein) in liver, kidney and gills of C. idellus on exposure to chlorpyrifos.

<table>
<thead>
<tr>
<th>Organ</th>
<th>15 days</th>
<th>30 days</th>
<th>60 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>10.9±</td>
<td>10.45±</td>
<td>10.03±</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>0.06*#</td>
<td>0.11*#</td>
</tr>
<tr>
<td>Kidney</td>
<td>6.45±</td>
<td>6.35±</td>
<td>6.21±</td>
</tr>
<tr>
<td></td>
<td>0.35</td>
<td>0.03*</td>
<td>0.05*</td>
</tr>
<tr>
<td>Gills</td>
<td>5.67±</td>
<td>5.29±</td>
<td>5.22±</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>0.005*</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Data is presented as Mean ± S.D., n = 6.

*p< 0.05, significant difference: control vs. 1.44 µg/L and 2.41 µg/L CPF treated group.

#p< 0.01, significant difference: control vs. 1.44 µg/L and 2.41 µg/L CPF treated group.

In kidney of the fish, the level of GSH decreased (1.55% and 3.72%) significantly (p< 0.05) form control after 15 days exposure to CPF at 1.44µg/L and 2.41 µg/L respectively. On 30th day exposure, GSH level showed significant (p< 0.05) decrease (5.65%) from control at lower concentration of the toxicant, though the change (7.97%) has found to be insignificant on exposure of the fish to higher concentration of the toxicant. Similar significant (p< 0.05) decrease in GSH level (6.3%) in kidney of the fish after 60 days exposure has been found at lower concentration of the toxicant, and at higher concentration the decrease (8.4%) has been found to be insignificant (Table 4). In gills, on exposure of the fish to chlorpyrifos for 15, 30 and 60 days, significant (p< 0.05) decrease in GSH level (6.70%, 7.44% and 10.35%) has been found at lower concentration of the toxicant, though the decline (7.93%, 10.28% and 14.4%) has been found to be insignificant at higher toxicant concentration (Table 4).

During present investigation, significant decrease in GSH level observed in liver, kidney and gills of C. idellus at different exposures could be due to its utilization to challenge the prevailing oxidative stress under the influence of ROS generated from pesticide exposure. Reduced GSH and its metabolizing enzyme provide the foremost defense against ROS induced cellular damage [47]. This decrease might be because of increased utilization of GSH, which can be converted to oxidized glutathione and potentially weak GSH regeneration. Further, they stated that GSH depletion indicates its exhaustion phase II biotransformation, thereby enhance the risk of oxidative stress due to reduced cell protection activity [45]. During present investigation, among the tissues studied, depletion in GSH was found to be highest in gills than liver and kidney and thus resulted in cell degeneration [57]. Similar decrease in liver & gills of Cyprinus carpio [58] & Carassius auratus [53] have also been reported on exposure to pesticide. Present

Observations are in concurrence with the findings of workers [46] studied effect of propiconazole on Oncorhynchus mykiss. They explained that replenishment of GSH in extra-hepatic tissue could be more difficult, so gills could serve as better biomarker of pollution. Contrary to the present findings, [44] while working on deltamethrin exposed Channa punctatus and on cypermethrin exposed C. punctatus reported an increase in GSH level in liver, kidney and gills of the fish and pointed it to be a primary protective response of the cell against oxidative stress induced by pollutants. Similar increase in GSH level has also been reported in liver and kidney of teleost fish exposed fish & explained that GSH undergoes oxidation after being conjugated in redox process and is converted to its reduced form as an adaptive response.

**Glutathione-s-transferase (GST)**

GST is a group of multifunctional isoenzymes, which play an important role in detoxification of toxic electrophiles by catalyzing the conjugation of a wide variety of electrophilic substrates to GSH and thus protects the cell from oxidative stress. It is considered as first line of defense against oxidative stress injury, decomposing superoxide radicals and hydrogen peroxide before interacting to form the reactive hydroxyl, which has a number of adverse biological effects when present in high amounts [60]. The activity of GST in liver showed a fluctuating trend throughout the exposure period. On 15th day, it showed significant (p<0.01) increase (0.92%) from control at 1.44 µg/L of CPF and at 2.41 µg/L CPF insignificant increase in GST activity (2.3%) has been observed. Whereas, on 30th day, significant (p<0.05) decrease in GST activity (0.45%) at lower concentration of the toxicant has been noticed, though the change (3.2%) has been found to be insignificant at higher concentration. After 60 days exposure, similar significant (p<0.05) reduction in GST activity (4.6%) has been seen at lower concentration of the toxicant, and at higher concentration the marked decrease (11.38%) observed has been found to be insignificant. All the changes observed in GST activity in liver of the fish have been found to be significant at lower concentration of the toxicant (Table 5). In kidney of the treated fish, the activity of GST showed significant (p<0.05) increase (5.8%) from control at higher concentration of the toxicant after 15 days exposure, though at lower concentration insignificant change (2.19%) occurred. Further, on 30th day exposure, significant (p<0.05) increase in GST activity (19.39%) at higher concentration has been noticed, and at lower concentration the elevation in GST activity (7.10%) has been found to be insignificant. After 60 days exposure, significant (p<0.05) elevation (3.7% and 12.85%) from control has been observed at lower and higher concentration of the toxicant respectively [Table 5]. In gills of C. idellus exposed to CPF for 15 days, significant (p<0.01) marked increase (12.32%) in GST activity has been noticed at higher concentration, though the elevated activity (6.78%) observed has been found to be insignificant at lower concentration of the toxicant. Similarly, on 30th day exposure, GST activity showed significant (p<0.05) increase (7.5%) at lower concentration and at higher concentration of the toxicant the change (8.3%) has been found to be insignificant. After 60 days exposure, GST activity showed significant (p<0.05) increase (2.6% and 6.14%) at 1.44 µg/L and 2.41 µg/L CPF respectively from control (Table 5). During present studies, the activity of GST in gills of CPF exposed fish was found to increase both with increase in exposure period and toxicant concentration. Similar observations have been made in other fishes (Oreochromis niloticus & Cyprinus carpio exposed to 2,4-D & azinophosmethyl [47] Brycon cephalus exposed to methyl parathion [45], Labeo rohita exposed to malathion [61], Danio rerio exposed to atrazine [33]. This, as explained [44], the increased GST activity in gills may indicate the development of a defensive mechanism to counteract the effects of pesticide and may reflect the possibility of a more efficient protection against pesticide toxicity. This suggests increased detoxification process.

**Table 5: Variation in GSH (µmoles/min/mg protein) in liver, kidney and gills of C. idellus on exposure to chlorpyrifos.**

<table>
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</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>1.44 µg/L</td>
<td>2.41 µg/L</td>
</tr>
<tr>
<td>Liver</td>
<td>10.9±</td>
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<tr>
<td></td>
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<tr>
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<td>0.35</td>
<td>0.035*</td>
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<tr>
<td>Gills</td>
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<tr>
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Data is presented as Mean ± S.D., n = 6

*p< 0.05, significant difference: control vs. 1.44 µg/L and 2.41 µg/L CPF treated group*  
#p< 0.01, significant difference: control vs. 1.44 µg/L and 2.41 µg/L CPF treated group

Contrary to the present findings, [43,46] other workers reported a decrease in GST activity. This could be attributed to short exposure of the toxicant (24-48 hr). In the kidney of the pesticide treated fish at all the exposures, an increase in the GST activity has been observed. Induced GST activity indicates the role of enzyme in protection against the toxicity of xenobiotics induced lipid peroxidation [44,47 & 62]. Whereas during acute exposure studies, no significant change in GST activity in kidney has been reported (Prochilodus lineatus exposed to atrazine [63], Carassius auratus exposed to carbamate fungicide [64]. Carassius
Oxidative Stress Response in Liver, Kidney and Gills of Ctenopharyngodon idellus (Cuvier & Valenciennes) Exposed To Chlorpyrifos

auratus exposed to sencor [34]. During present investigation, GST activity in the pesticide exposed fish found to increase on 15th day exposure and then showed a continuous decrease up to 60 days. Initial increase in the GST activity has also been reported by other workers [61-64]. As observed during present investigation with increase in exposure period inhibition in GST activity have also been reported in other fishes (Carassius auratus exposed to 2, 4-D [53], Oreochromis niloticus exposed to oxyfluorfen [65], Oreochromis mykiss exposed to diazinon & methyl parathion [66].

The increased GST activity is concomitant to the decrease in GSH level in liver and gills [45]. From the above discussion, it could be inferred that GST utilizes GSH for the xenobiotic detoxication.

Correlation coefficient among oxidative stress parameters

In gills of chlorpyrifos treated fish, a negative correlation has been found between GSH and GST (r= 0.896, p < 0.01), and a positive correlation between LPO and GSH (r= -0.706, p < 0.01) at 1.44 µg/L chlorpyrifos. Whereas on exposure to 2.41 µg/L chlorpyrifos, there was a positive correlation between GSH and CAT (r= 0.762, p < 0.01), and a negative correlation between LPO and GSH (r= -0.76, p < 0.01), and LPO & catalase (r= -0.870, p < 0.01). In the liver, a positive correlation has been found between LPO & GSH (r= 0.730, p < 0.01), and a negative correlation between LPO and GST (r= -0.654, p < 0.01) at lower toxicant concentration. Whereas at higher toxicant concentration, a positive correlation between GSH and LPO (r= 0.730, p < 0.01), and a negative correlation between GST and GSH (r= -0.520, p < 0.01) has been found. In the kidney, a positive correlation between SOD and LPO (r= 0.794, p < 0.01), and a negative correlation between LPO and GST (r= -0.807, p < 0.01), GST & GSH (r= -0.794, p < 0.01), SOD & GST (r= 0.643, p < 0.05) has been observed at 1.44 µg/L of chlorpyrifos. Whereas at higher toxicant concentration, positive correlation between SOD and CAT (r= 0.798, p < 0.01), LPO and CAT (r= 0.725, p < 0.01), and negative correlation between GSH & CAT (r= -0.714, p<0.01) has been found. The significant correlation found between antioxidants in C. idellus might enforce to initiate the detoxifying system in fighting against chlorpyrifos induced oxidative stress [67]. Thus, striking strong correlation found between some variables suggested that the fish, C. idellus would have been enforced to initiate the detoxifying system in fighting against chlorpyrifos toxicity and induced oxidative stress [67].

Multivariate analysis

To explore overall relationships between the different oxidative indices, PCA from component matrix data was made. The results showed that on exposure of the fish to the toxicant, 90.45% of overall variance was explained by the first two principal components in liver. The principle component I (71.41% of variance) was built by LPO, GSH, GST, CAT & SOD. The principle component II (19.04% of variance) was formed by CAT. Vector plot made on the basis of PCA, showed that there was a relationship between GSH, GST & SOD (Figure 1). In kidney 93.91% of overall variance was explained by the first three principal components. The first principle component (59.21% of variance) was built by LPO, GSH, GST, CAT & SOD. The principle component II (22.33% of variance) was formed by CAT, LPO, GSH & GST. The principle component III (12.38% of variance) was formed by CAT, LPO &GST. Vector plot made on the basis of PCA, showed that there was a relationship between CAT & SOD (Figure 2). In gills, 92.45% of overall variance was explained by the first two principal components. The first principle component (83.27% of variance) was built by LPO, GSH, GST, CAT & SOD. The principle component II (9.18% of variance) was formed by GST & SOD. Vector plot made on the basis of PCA, showed that there was a relationship between GSH & GST (Figure 3).
Conclusion

From the present findings, it is concluded that oxidative stress may be attributed to chlorpyrifos induced hepatic, renal and gill toxicity. The result of such exposure lead to oxidative stress might have impaired cellular function which can lead to certain diseases or may cause death. CPF was found to be highly toxic to Ctenopharyngodon idellus even at very low concentration. Its administration promotes MDA level and affected adversely the antioxidative defense system in various organs of the fish. The parameters measured could provide useful information for evaluating the toxicological effects of chlorpyrifos on the fish and help in the diagnosis of the pollution.

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References


