PESTICIDES MIXTURE TOXICITY; EFFECTS ON SUPEROXIDE DISMUTASE ACTIVITY IN INDIAN MAJOR CARPS

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INTRODUCTION

Due to rapid increase in the industrialization and human population, the pollution in aquatic ecosystem has become an universal phenomenon in the present day world (Belazutshi and Raghuprasad, 2008). The main sources of water pollution are industrial waste, domestic sewage, drainage and agriculture effluent which contain pesticides (Maruthanayagam and Sharmila, 2004). These pesticides are too toxic and have adverse effects on aquatic organisms and water quality (Barbieri and Ferreira, 2011). Acute toxicity refers to the toxicant’s ability to cause harm to an organism from a single exposure, commonly of short period. Mostly the acute toxicity test of pesticides has been used for rapid estimation of the concentrations that cause direct and irreversible damage to the fish (Pandey et al., 2005). Acute lethality and LC50 are most common acute toxicity tests which signify the lethality of test organisms in terms of mortality and exposure duration (Karra et al., 2015). Pesticides are able to disturb the fish organs, nervous system, reproduction, hormone regulation, embryonic development and growth at non-lethal dose (Palma et al., 2008 and 2009a,b).

Most common environmental pollutant entering the aquatic ecosystem is endosulfan, an organochlorine pesticide causing mass mortality of fish. It is highly stable and accumulates in the food web (EJF, 2002; Pandey et al., 2006). In the beginning of 1980’s organophosphate (OP) pesticides has been extensively used in place of organochlorine (Svoboda et al., 2001). Most preferable organophosphorus is chlorpyrifos, because of their low cumulative ability and lower persistence in the environment but has adverse effects on aquatic life (Spalving and Fellers, 2007). Organochlorine and organophosphorus pesticides have been fully replaced by synthetic pyrethroid (Tiwari and Ansari, 2014) due its high selectivity and short term persistence in the environment (Goulding et al., 2013). Bifenthrin belong to pyrethroids pesticides those are extremely dangerous to fish because at the nerve cell ending it interfere with Na+ channel gating as well as with other ions channels like Cl− and Ca2+ (Burr and Ray, 2004). Pesticide stimulated oxidative stress has become increasingly reported from the last few decades in toxicological research as a possible mechanism of toxicity (Ciccheti and Argentin, 2003; Sharma et al., 2005). To save the biomolecules from the injurious effects of reactive oxygen species (ROS) resulting from the breakdown of different toxicants, fish possess an antioxidant defense system. Antioxidant defense system of fish consist of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione S-transferase (GST) and glutathione reductase (GR) (Mate, 2000). First defensive mechanism in fish against oxidative stress is SOD; facilitate dismutation of superoxide radicals to H2O2. Therefore, this can be used as biomarker for ROS production in pesticide toxicity (Kohen and Nyska, 2002). Species-specific response to toxicants is of great importance, because it is a key feature estimating toxicity as well. A lot of information on freshwater organisms was able to document a few of these species-specific sensitivities to various contaminants (Pyle and Wood, 2008; Schjolden et al., 2007).
mrigala and Labeo rohita are the common freshwater fishes of south-east Asian countries. Information regarding the 96-hr LC₅₀ and lethal toxicity of pesticides mixture in L. rohita, C. catla and C. mrigala has not been reported till date. Therefore, this study was conducted to determine the 96-hr LC₅₀ and lethal toxicity of pesticides mixture (Bifenthrin+Chlorpyriphos+Endosulfan) to Indian major carps and its effect on oxidative stress biomarker such as superoxide dismutase activity in the liver of three fish species.

MATERIALS AND METHODS

Acute toxicity test: The experimental fish species (Catla catla, Cirrhina mrigala and Labeo rohita) were purchased from Fish Seed Hatchery, Faisalabad, Pakistan. The acute toxicity tests (96-hr LC₅₀ and lethal concentration) were conducted in the wet laboratory at Fisheries Research Farms, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan. Before the start of the experiment, fish were kept in cemented tank for acclimatization for two weeks. During this period fish were fed with commercial feed at 3% wet body weight. All aquaria (70-L) were well aerated with an air pump and filled with dechlorinated tap water and stocked with fish. Stock solutions of pesticides viz. Bifenthrin (BIF), Chlorpyrifos (CPF) and Endosulfan (END) were made in methanol while pesticides mixture (Bifenthrin+Chlorpyrifos+Endosulfan) of required concentration were made by further dilutions in the deionized water. The acute toxicity tests were replicated three times. During experimental trials, water pH, temperature and total hardness were maintained at 7.5, 30°C and 250 mgL⁻¹, respectively. The physico-chemical parameters were monitored on daily basis by following standard methods (A.P.H.A. 1998). Mortality was recorded during 96-hr after the start of the tests and dead fish were removed immediately.

Superoxide dismutase activity: After determination of 96-hr LC₅₀ value of Bifenthrin + Chlorpyrifos + Endosulfan mixture sampling was done at interval of 24, 48, 72 and 96-hr to evaluate the superoxide dismutase activity in the liver of all three fish species.

Preparation of homogenate: To remove the RBCs, the dissected organ i.e. liver was rinsed with phosphate buffer of 0.2 M (pH 6.5) and homogenized in cold buffer (1:4 w/v). Homogenate organ was centrifuged at 10,000 rpm for 15 minutes at 4°C. After this, clear supernatant was separated for enzyme assay while residue was discarded.

Enzyme Assay: The activity of superoxide dismutase was determined by measuring its ability to inhibit the photoreduction of Nitrobluetetrazole (NBT) by following the method of Giannopolitis and Ries (1977). Reaction mixture contained 1 ml of 0.067 mM phosphate buffer (pH 7.8), 0.016 ml of riboflavin and 0.05 ml enzyme extract. This mixture was kept in light box with an internally mounted light bulb of 30 Watt for 12 minutes. After that 0.067 ml of EDTA/NaCN solution and 0.033 ml of NBT was added to the illuminated reaction mixture and absorbance was noted after 20s of reaction by spectrophotometrically at A₅₅₀ nm against blank (buffer).

Statistical analyses: The whole experiment was performed with three replicates. The 96-hr LC₅₀ and lethal concentrations of pesticides mixture for all three fish species were determined by analyzing the fish mortality data through Probit Analysis method (Hamilton et al., 1977) at 95% confidence interval. Analysis of variance and comparison of means were performed by using Tukey’s/Student Newman-Keul tests to find-out statistical differences among different variables under study.

RESULTS AND DISCUSSION

Acute toxicity test: The acute toxicity data with mean 96-hr LC₅₀ and lethal concentration (μgL⁻¹ ±SD) values, duration of exposure, concentration of pesticides in mixture, 95% lower and upper confidence interval limits with their calculated chi-square values are given in Table 1. The calculated chi-square (χ²) with higher p-values (0.992-1.000) showed higher precision of all regression models. Results indicated that among all three fish species, C. catla showed

<p>| Table 1. The 96-hr acute toxicity of pesticides mixture (μgL⁻¹±SD) for Indian Major Carps. |</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mixture Ratio</th>
<th>Fish species</th>
<th>LC₅₀ (μgL⁻¹)</th>
<th>95% C.I. LCL-UCL</th>
<th>Lethal conc. (μgL⁻¹)</th>
<th>95% C.I. LCL-UCL</th>
<th>Pearson Goodness of Fit Tests</th>
<th>Regression equation (Y=a+bx)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIF + CPF + END</td>
<td>1:1:1</td>
<td>C. catla</td>
<td>1.09±0.01c</td>
<td>0.906-1.218</td>
<td>1.9±0.02c</td>
<td>1.693-2.305</td>
<td>1.043</td>
<td>8</td>
<td>0.998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C. mrigala</td>
<td>1.81±0.02a</td>
<td>1.635-1.960</td>
<td>2.78±0.02a</td>
<td>2.532-3.266</td>
<td>2.392</td>
<td>10</td>
<td>0.992</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L. rohita</td>
<td>1.63±0.02b</td>
<td>1.477-1.741</td>
<td>2.30±0.03b</td>
<td>2.127-2.632</td>
<td>0.699</td>
<td>8</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Means with the same letters (small) in a single column are statistically similar at p<0.05. BIF=Bifenthrin; CPF= Chlorpyriphos; END=Endosulfan; C. catla = Catla catla; C. mrigala=Cirrhina mrigala; L. rohita=Labeo rohita; C.I.= Confidence Interval (μgL⁻¹); LCL=Lower Confidence Limit (μgL⁻¹); UCL= Upper Confidence Limit (μgL⁻¹); Lethal Conc., Lethal Concentrations (μgL⁻¹); χ² = Chi-Square; DF= Degree of Freedom; y=Dependent variable; x=Independent variable; value within the bracket is the standard error; **= Significant at p<0.01

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more sensitivity toward BIF+CPF+END mixture with mean 96-hr LC$_{50}$ and lethal concentration of 1.09±0.01 and 1.9±0.02 μgL$^{-1}$, respectively. The deviance chi-square and p-value for *C. catla* was calculated as 1.043 and 0.998, respectively. The mean 96-hr LC$_{50}$ and lethal concentrations of tertiary mixture of pesticides (BIF+CPF+END) were ranged from 1.63±0.02-2.30±0.03 for *L. rohita* while it was estimated as 1.81±0.02 and 2.78±0.02 μgL$^{-1}$, respectively for *C. mrigala*. It showed higher tolerance limit against tertiary mixture of pesticides. The sensitivity of all fish species varied significantly under the exposure of tertiary mixture of pesticides (BIF+CPF+END). The sensitivity of three fish species toward pesticides mixture was increased in the following order: *C. catla>*L. rohita>*C. mrigala*. Our results are also supported by Ilyas and Javed (2013) who performed acute toxicity test with three fish species viz. *C. catla*, *C. mrigala* and *L. rohita* exposed to endosulfan. The 96-hr LC$_{50}$ values of endosulfan for these major carps were calculated as 0.98, 1.06 and 2.15 μgL$^{-1}$, respectively which shows that the *C. catla* is more sensitive than other species. According to Al-Rudainy and Kadhim (2012), toxicant concentration and exposure duration are the main factors responsible for fish mortality. Ambreen and Javed (2015) reported the acute toxicity of pesticide mixture (CPF+END+BIF) to the fish, *Cyprinus carpio* and *Ctenopharyngodon idella*. The mean 96-hr LC$_{50}$ and lethal concentrations of this mixture were 0.22±0.01 and 0.32±0.02 μgL$^{-1}$, respectively for *C. carpio* while the mean 96-hr LC$_{50}$ and lethal concentrations were ranged from 2.16-7.49 μgL$^{-1}$ and 3.86-13.86 μgL$^{-1}$, respectively for *C. idella*.

### Superoxide dismutase activity

In the present study, it was found that the activity of SOD in the liver of all three fish species exposed to tertiary mixture of pesticides was significantly (p<0.05) higher as compared to control (Table 1). Comparison among fish species showed that *C. catla* had higher SOD activity as compared to other species exposed to tertiary mixture (Table 2). Regression equation for enzyme activity is given in Table 3 which shows the dependence of SOD activity on exposure time was significantly positive with R$^2$ value up to 0.991, 0.976, and 0.99 for *C. catla*, *C. mrigala* and *L. rohita*, respectively. It

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**Table 1. Relationship between the concentration of mixture and mortality of *C. mrigala*, *L. rohita* and *C. catla* during 96-hr acute toxicity.**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Control</th>
<th>Treated</th>
<th>Control</th>
<th>Treated</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>59.11±0.34a</td>
<td>103.20±0.49d</td>
<td>38.15±0.18a</td>
<td>80.35±4.59d</td>
<td>47.51±0.11a</td>
<td>99.96±0.25d</td>
</tr>
<tr>
<td>48</td>
<td>59.12±0.35a</td>
<td>119.75±0.48c</td>
<td>38.17±0.26a</td>
<td>100.42±6.40c</td>
<td>47.52±0.17a</td>
<td>115.21±0.34c</td>
</tr>
<tr>
<td>72</td>
<td>59.14±0.40a</td>
<td>131.08±0.28b</td>
<td>38.18±0.27a</td>
<td>119.17±10.23b</td>
<td>47.54±0.12a</td>
<td>125.05±0.45b</td>
</tr>
<tr>
<td>96</td>
<td>59.15±0.42a</td>
<td>148.67±0.65a</td>
<td>38.19±0.35a</td>
<td>130.05±7.12a</td>
<td>47.55±0.12a</td>
<td>140.05±0.29a</td>
</tr>
</tbody>
</table>

Means with similar letters in a single row are statistically similar at p<0.05 ANOVA followed by HSD Tukey test.

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**Table 2. The SOD activity (UmL$^{-1}$±SD) in liver of three fish species exposed to BIF+CPF+END mixture for different time intervals.**

<table>
<thead>
<tr>
<th>Fish Species</th>
<th>Exposure Durations</th>
<th>24-hr</th>
<th>48-hr</th>
<th>72-hr</th>
<th>96-hr</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. catla</em></td>
<td></td>
<td>103.20±0.49d</td>
<td>119.75±0.48c</td>
<td>131.08±0.28b</td>
<td>148.67±0.65a</td>
</tr>
<tr>
<td><em>C. mrigala</em></td>
<td></td>
<td>80.35±4.59d</td>
<td>100.42±6.40c</td>
<td>119.17±10.23b</td>
<td>130.05±7.12a</td>
</tr>
<tr>
<td><em>L. rohita</em></td>
<td></td>
<td>99.96±0.25d</td>
<td>115.21±0.34c</td>
<td>125.05±0.45b</td>
<td>140.05±0.29a</td>
</tr>
<tr>
<td>Overall Means</td>
<td></td>
<td>94.5±12.36D</td>
<td>111.79±10.11C</td>
<td>125.10±5.96B</td>
<td>139.59±9.32A</td>
</tr>
</tbody>
</table>

Means with similar letters in a single row and *column are statistically similar at p<0.05 ANOVA followed by HSD Tukey test.

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### Figure 1

Figure 1. Relationship between the concentration of mixture and mortality of *C. mrigala*, *L. rohita* and *C. catla* during 96-hr acute toxicity.
Table 4. Relationship between exposure time of BIF+CPF+END mixture and SOD activity (UmL⁻¹) in liver of three fish species.

<table>
<thead>
<tr>
<th>Fish Species</th>
<th>Regression Equation</th>
<th>SE</th>
<th>r</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. catla</td>
<td>88.7 + 0.616 **Time (hr)</td>
<td>0.03451</td>
<td>0.995</td>
<td>0.991</td>
</tr>
<tr>
<td>C. magina</td>
<td>65.5 + 0.699 **Time (hr)</td>
<td>0.06355</td>
<td>0.988</td>
<td>0.976</td>
</tr>
<tr>
<td>L. rohita</td>
<td>87.5 + 0.542 **Time (hr)</td>
<td>0.03119</td>
<td>0.994</td>
<td>0.990</td>
</tr>
</tbody>
</table>

SE=Standard Error; r= Multiple Regression Coefficient; Coefficient of Determination; **=Highly significant at p<0.01

was observed that the SOD activity showed time dependent increasing trend in the liver of all three fish species. These findings are also supported by Ullah et al. (2016) who observed a time dependent elevation in superoxide dismutase activity in the liver of L. rohita exposed to endosulfan.

Alteration in enzymes activities and metabolic functions by increased reactive oxygen radicals is considered as the fish response to toxicant exposure (Oruc et al., 2004; Isik and Celik, 2008). The induction in the SOD activity under oxidative stress is considered as a first line of defense provide an important adaptation against toxicants produced stress (Gultekin et al., 2000). In addition, liver is a key organ responsible for detoxification, metabolism and excretion of toxicants from the body (Hinton and Lauren, 1990). According to Mohamed (2009) the liver is also a target organ due to its large blood supply which causes noticeable toxicant exposure.


Hamilton, M. A., R.C. Russo and R.V. Thurston. 1977. Trimmed spearmen-Karber method for media lethal...


