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Research Article

Assessment of Pesticides Pollution in Water by Studying Biochemical and Molecular Parameters in Fish

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Abstract: Enzymatic antioxidants serve as an important biological defense against oxidative stress. Information on antioxidant defense in fish is meager despite that fish are constantly exposed to environmental stress. Therefore, this study was planned to assess the changes in biochemical and molecular parameters in various tissues of freshwater fish, *Ctenopharyngodon idella*., in response to long-term exposures to pesticides mixture. Fish were exposed to different sub-lethal concentrations (1/5th of LC_{50}, 1/4th of LC_{50}, 1/3rd of LC_{50}) of pesticides mixture for 60 days. The samples of fish blood and organs *viz*. gills, liver, kidney, brain, muscles and heart were dissected after every 15 days of exposure to assess the catalase activity and DNA damage. Result of this study revealed that exposure of pesticides mixture result of induced significant decrease in catalase activity in all selected organs of test fish with the increase in exposure duration and the mutagenic potential of the pesticides causes maximum DNA damage after 60 days of pesticides mixture exposure. An exposure of 1/3rd of LC_{50} to *C. idella* induced significantly higher number of nuclear abnormalities in-terms of dumble, blebbed and notched shaped nuclei. The variation in biochemical and molecular variation parameters in different organs of test fish samples in comparison with the control shows the adverse effect of pesticides pollution on the fish health.

Keywords: Freshwater fish, catalase activity, genotoxicity, biochemical parameters, pesticides mixture

1. INTRODUCTION

Environmental pollution by toxicants has become one of the most important problems in the world [1]. Pesticides constitute the major source of potential environmental hazards not only for animals but also to human when they become part of food chains [2]. Intensive agriculture activities require the application of large quantities of pesticides annually. The long term application of these pesticides is always expected to induce pesticide residues accumulation in soil, water, and in the general environment, thereby posing a serious threat to public health [3]. It is believed that numerous undocumented cases of sub-lethal poisoning of pesticides to fish, due to direct dermal contact or ingestion of contaminated food and water occur annually [4]. An effective monitoring system using biochemical and molecular markers has been established to demonstrate these pesticides in the environment. Long term exposure to pesticides mixture causes countless abnormalities and reduces the life span of aquatic organisms [5]. Fish are more frequently exposed to these pollutants because it is believed that regardless of where the pollution occurs, it will eventually end up in the aquatic environment.

In this study, we focused on three (3) pesticides (Endosulfan, chlorpyrifos and Bifenthrin). Among all forms of chemical pesticides, organochlorines are considered to be the most dangerous with reference to environmental pollution, since they are very tenacious and non-biodegradable and add to the residue accumulation in the food chain. Endosulfan is one of organochlorines and is highly toxic and potentially bio-accumulative for fish USEPA [6].Organophosphates (OP) is exploited intensively due to their low accumulative capacity and short-range persistence in the environment. Organophosphate pesticides are extremely lethal to fish even at commend levels due to their

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resolution in the environment and bioaccumulation in the several organs of fish. Among OP pesticides, chlorpyrifos (CPF) is extensively used. CPF toxicity in fish leads to oxidative stress and DNA damage. The lethal effects of chlorpyrifos (CPF) are progressively threatening the health of aquatic biota [7]. It may induce oxidative stress and inhibit anti oxidative and physiological activities [8]. Pyrethroids are amongst the most frequently used pesticides all over the world, and pose a danger to the natural environment, including aquatic biota [9]. Many pyrethroids may have potentially toxic effects at sub-lethal levels. Bifenthrin (BF) is one of the pyrethroids pesticides and is normally used in agriculture, but a small number of studies for the toxicity of BF on fishes are accessible. Bifenthrin is a newly commenced type-I pyrethroid with eight stereoisomer's, of which the cis-isomer is the vigorous element [10]. Pesticides are one of the major contributors to oxidative stress [11]. Oxidative stress occurs when the critical balance between oxidants and antioxidants is disrupted due to the depletion of antioxidants or excessive accumulation of the reactive oxygen species (ROS), or both, leading to damage [12].

Pesticide accumulation in tissues has been associated with increased oxidative stress and production of ROS [13]. Moreover, many of these potentially toxic compounds or their metabolites have shown toxic effects related to oxidative stress in fish [14]. One of the main antioxidant enzymes that assist to detoxify reactive oxygen species is catalase (CAT). CAT is a ubiquitous heme protein that degrades hydrogen peroxide (H_2O_2) to oxygen and water and is recognized as the important biological marker to produce oxidative stress before the occurrence of significant effects in fish [15]. Pesticides have been reported to lead to DNA damage which appears in the form of micronucleus formation, chromosome aberrations and mitotic aberrations [16]. Micronucleus appearance in the cytoplasm is considered as biomarker of DNA damage [17].

Micronuclei are of same color, refraction and texture to that of nucleus and appear as separate small nuclei having size of $1/10^{th}$ in length and $1/3^{rd}$ in diameter of the main nucleus. With the treatment of pyrethroids, micronucleus could result when the entire or chromosome fragments are not incorporated in the main nucleus after cell division [18]. As a result of genetic damage, i.e., damage to the chromosomes, fragments lagging in the course of anaphase or lagging acentric chromosomes or cytoplasmic chromatin-containing bodies are failed to be incorporated into daughter nuclei (clastogenesis), results in the development of micronuclei in red blood cells [19]. Due to exposure of these pesticides, micronucleus formation, sister chromatids or chromosomal aberrations have been documented [20]. Among the aquatic species, the fish are the major targets of toxicants contamination. Fish are largely being used for the assessment of the quality of aquatic environment and as such can serve as bioindicators of environmental pollution.

The grass carp *Ctenopharyngodon idella*, a member of the family Cyprinidae, plays an important role in carp polyculture systems in Asia. Fish are frequently used as bio indicator since they are sensitive to changes in their environment and play a significant role in assessing potential risks associated with contamination [21]. Fish occupy the upper level in aquatic food chain and are susceptible to the presence of contaminants, including pesticides. They can therefore be regarded as important indicators of environmental pollution [22].

The primary aim of present study was (1) to evaluate the toxicity produced at biochemical and molecular level, with continuous intoxication with pesticides mixture for two months, on various biochemical parameters of tissues, such as liver, muscle, brain, kidney and heart in *C. idella*. (2) To assess the DNA damage at molecular level to *C. idella* caused by toxication of pesticides mixture, and (3) To monitor the Catalase activity as potential bio-marker to reduce oxidative stress in *C. idella* due to contamination of pesticides.

2. MATERIALS AND METHODS

The fingerlings of experimental fish, *Ctenopharyngodon idella* were purchased from Fish Seed hatchery, Faisalabad. Prior to the experiment, *C. idella* fingerlings were acclimatized to laboratory conditions for two weeks.After acclimation period, fish were transferred to 70-liter glass aquarium for experimental studies.

2.1 Chemicals

The technical grade chlorpyrifos ($C_9H_{11}C_{l3}NO_3PS$; 98% pure), endosulfan ($C_9\ H_6\ Cl_6\ O_3\ S$; 98.65 % pure) and bifenthrin ($C_{23}\ H_{22}\ ClF_3O_2$; 98.5% pure) were dissolved, separately, in 95 % analytical grade methanol (J.T Baker) as a carrier solvent to prepare the Stock-I solutions (1g/100ml) while tertiary mixtures of pesticides were prepared by its further dilutions in deionized water (stock-II).

2.2 Experimental Design

Glass aquaria of 70 liter water capacity were used to carry out the chronic toxicity test. The aquaria were washed thoroughly and filled with 40 liter dechlorinated tap water. The fish fingerlings were selected for chronic toxicity test of pesticides mixture. Ten fish of *C. idella* were kept in each aquarium to test different concentrations of pesticides mixture i.e. $1/5^{th}$, $1/4^{th}$ and $1/3^{rd}$ of LC_{50}. Each concentration was tested with three replications.Exposure media were renewed and maintained with desired concentration after every 24 hours to prevent any lowering of exposed concentration.

The water temperature (30ºC), pH (7.0) and total hardness (200 mg L^{-1}) were kept constant throughout the study period. However, calcium, magnesium, sodium, potassium, total ammonia, carbon dioxide and electrical conductivity were measured on daily basis by following the method described in "A. P. H. A [23]. Continuous air was supplied to all the test and control media with an air pump through capillary system. Continuous air was supplied to all the test and control media with an air pump through capillary system.

2.3 Enzyme Activity Assay

During experimental period, the fish was dissected after every 15-day of exposure and organs *viz*. liver, kidney, brain, gills, heart and muscle were stored for enzyme analysis. Catalase activity was determined by its ability to decrease the H_2O_2 concentration at 240 nm by using the method of Chance and Mehaly [24].

2.4 Molecular Study

After sub-lethal chronic exposures of fish to different concentrations of pesticides mixture, the DNA damage in peripheral erythrocytes of fish was assessed by using Micronucleus test. The control fish (pesticide free water) was used as a negative control group (unstressed) as well as a positive control was also run. Cyclophosphamide (sigma) was used as positive control. The positive control fish were subjected to an intra-peritonea injection of cyclophosphamide in a 4% saline solution with a concentration of 20 µg g^{-1} body weight.

Micronuclei test was performed on exposed and control fish peripheral erythrocytes to observe the nuclear abnormalities. A drop of blood from the fish caudal vein was directly smeared on slide and air-dried. Smears were subsequently fixed in methanol for 10 minutes and stained with wright-giemsa stain for 8 minutes [25]. The frequency of micronuclei (MN), nuclear buds and bi-nucleated erythrocytes were evaluated (per 1000 cells) by scoring at a 1,000 X magnification by using a binocular microscope (LABOMEDCXR3) under oil emersions (100 X) lens.

A total of 2,000 erythrocytes with intact cellular and nuclear membranes were examined for each fish species. Blind scoring of micronuclei and other nuclear abnormalities of cell (dumble, notched and blebbed shape of cell nuclei) were performed on coded slides. In general, the colour intensity of MN was the same or lowers than that of the main nuclei using criteria described by Fenech et al [26]. MN frequency was calculated as follows:

2.5 Statistical Analysis

Data obtained was analyzed by appropriate methods of Statistics [27]. MS Excel and Slide write plus software were used to draw graphs.

3. RESULTS AND DISCUSSION

3.1 Enzyme Studies

The experimental fish exposed to different concentrations of pesticides mixture exhibited lower catalase activity in gills, liver, kidney, brain, muscle and heart as compared to control. Comparison among treatments showed significantly lowest mean catalase activity in fish exposed to $1/3^{rd}$ of LC_{50} followed by $1/4^{th}$ and $1/5^{th}$ of LC_{50} as compared to control. Among exposure durations, lowest mean catalase activity was noted in all the studied organs of 60-day exposed fish while the

highest was observed in organs of 15-day exposed fish (Figure 1).

Pesticides may induce oxidative stress, leading to the generation of free radicals and may be the underlying molecular mechanism that gives rise to pesticide induced toxicity. Antioxidant enzymes are part of the cellular defense mechanisms that limit the negative impact of oxidant molecules on tissues and protect against oxidative cell injury by scavenging free radicals. Their ability to eliminate reactive oxygen species could be hampered by deviation in physiological concentrations with the consequence of increased oxidative damage to cellular lipids, proteins, and DNA [28, 29]. Crestani et al. [30] for instance, observed a reduction in CAT activity in the liver of silver catfish exposed to the herbicide clomazone. Similar effects were found by Pandey et al. [30] in the liver of the freshwater fish *Channa punctatus* texposed with endosulfan. Blahová et al. [31] observed a significant decline in CAT activity in zebra fish exposed to atrazine this paper reviews the same results as decrease in catalase activity in liver to pesticides exposed *C. idella*.

Oruç and Usta [32] reported that diazinon caused a decrease in the CAT activity in the muscle tissue of *Cyprinus carpio*. Similarly, our results showed that CPF exposure caused significant decreases in the CAT activities in the brain, liver, gill and muscle tissues. Several enzymatic pathway alterations are also induced by pesticides pollutions in organisms. Torre et al [33] reported that *Cyprinus carpio* was highly sensitive to pollutant and showed reduce level of acetylcholine esterase. Under deltamethrin exposure, reduced level of ascorbic acid was observed in *Channa punctatus* [34, 35] studied the pesticide toxicity impacts in various tissues of *Cyprinus carpio* exposed to lethal concentrations of different pyrethroids and found decrement in SDH. Somnuek et al [36] analyzed the AChE activity in the brain, liver, muscle and gill tissues of hybrid catfish, *Clarias macro cephalus* and *Clarias gariepinus* exposed to a sublethal concentration of an organophosphate, chlorpyrifos and a carbamate, carbaryl and reported that AChE inhibition increased rapidly with pesticide concentration. Rapid AChE inhibition with insecticide concentration has also been observed under cypermethrin exposure in different fishes [37]. Many other studies of pesticides found as inducer of anomalous biochemical changes in fish [38].

3.2 Molecular Studies

The DNA damage was determined in-terms of micronuclei frequency and frequency of other nuclear abnormalities varied significantly due to exposure of various concentrations of pesticides mixture. Micronuclei frequency and occurrence of other nuclear abnormalities in the peripheral erythrocytes of *C. idella* exposed to pesticides mixture at different concentrations *viz*. $1/3^{rd}$, $1/4^{th}$ and $1/5^{th}$ of LC_{50} and compared with negative and positive controls. An exposure of $1/3^{rd}$ LC_{50} to *C. idella* induced significantly higher number of nuclear abnormalities in-terms of dumble, blebbed and notched shaped nuclei. The total frequency of other nuclear abnormalities in peripheral erythrocytes of *C. idella*, exposed to various concentrations of pesticides mixture was also significantly higher at $1/3^{rd}$ of LC_{50} followed by that of positive control and negative control (Table 1).

There is rich documented literature witnessing research on molecular level of different fish species showing ill-effects of pesticides on genes and DNA levels [39]. Due to chemical pollutants the low amount of DNA per cell, the large numbers of small chromosomes, and the low mitotic activity in many fish species impaired the metaphase analysis of chromosomal damage and sister chromatid exchanges are demonstrated [40].

Pesticides have been reported to lead to DNA damage which appears in the form of micronucleus formation, chromosome aberrations and mitotic aberrations [41]. Micronucleus appearance in the cytoplasm is considered as biomarker of DNA damage [42]. Sankar et al [43] reported that with the treatment of pyrethroids, micronucleus could result when the entire or chromosome fragments are not incorporated in the main nucleus after cell division. Sharaf et al [44] investigated in his studies that genetic damage, i.e., damage to the chromosomes, fragments lagging in the course of anaphase or lagging acentric chromosomes or cytoplasmic chromatin-containing bodies are failed to be incorporated into daughter nuclei (clastogenesis), results in the development of micronuclei in red

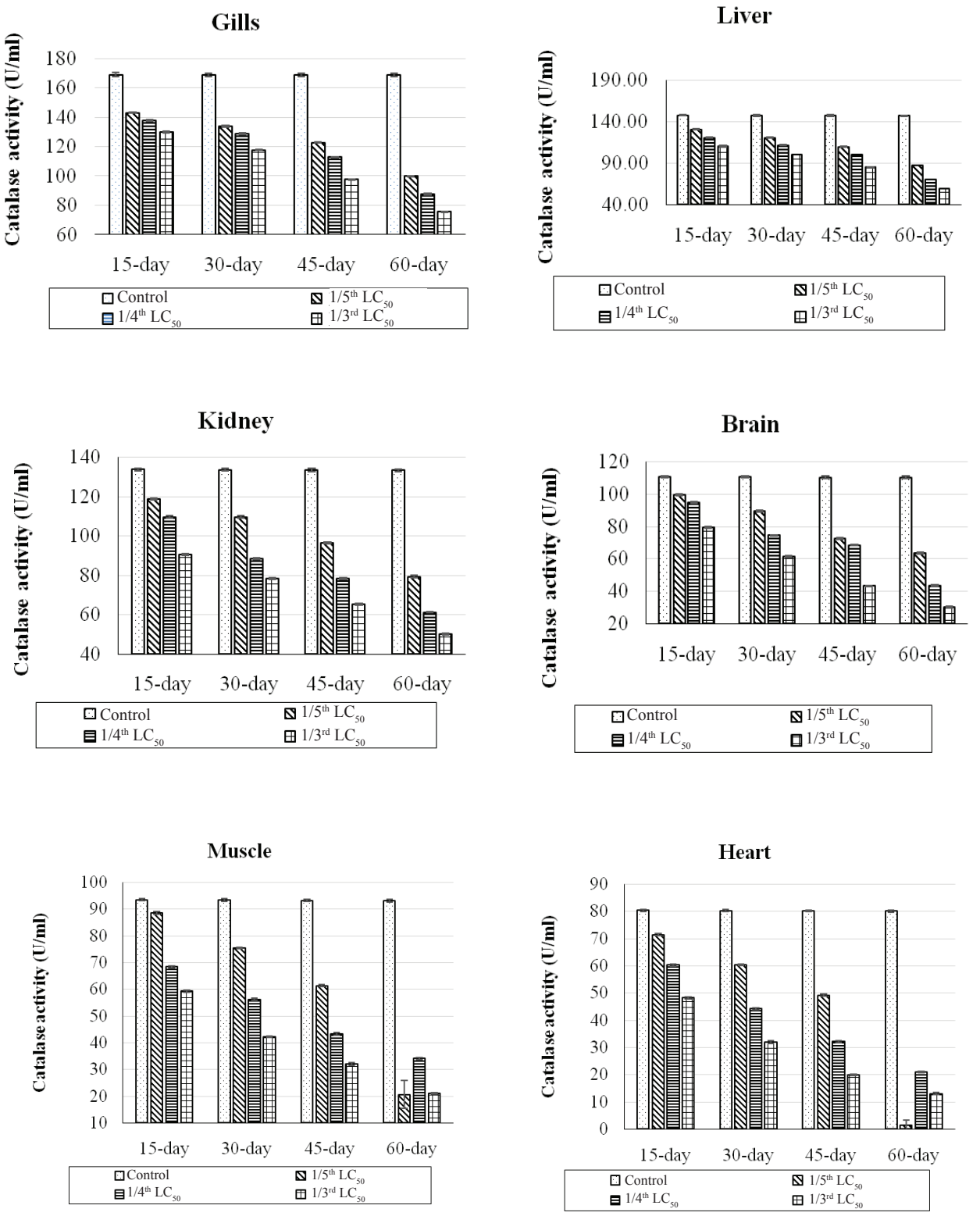

Fig. 1. Enzyme activity in different organs of fish exposed to pesticides mixture.

Table 1. Nuclear abnormalities in blood cells of fish exposed to pesticides mixture

Treatments	Abnormalities (%)				
	Exposure Durations	Dumble	Blebbed	Notched	Total frequency
Negative Control	15-day	0.40±0.05	0.00±0.00	0.00±0.00	0.40±0.03
	30-DAY	0.60±0.04	0.00±0.00	0.00±0.00	0.60±0.05
	45-day	0.30±0.04	0.00±0.00	0.00±0.00	0.30±0.05
	60-day	0.20±0.04	0.00±0.00	0.00±0.00	0.20±0.04
Positive control	15-day	0.50±0.04	0.23±0.05	0.21±0.04	1.03±0.03
	30-day	1.45±0.04	0.49±0.03	0.33±0.06	2.27±0.05
	45-day	0.32±0.04	0.18±0.03	0.07±0.02	0.57±0.03
	60-day	0.18±0.03	0.13±0.06	0.01±0.02	0.32±0.06
$1/5^{th}$ of LC_{50}	15-day	0.77±0.04	0.16±0.04	0.12±0.06	1.05±0.04
	30-day	1.20±0.02	0.28±0.03	0.17±0.06	1.65±0.03
	45-day	0.42±0.04	0.05±0.03	0.01±0.02	0.48±0.03
	60-day	0.21±0.07	0.01±0.02	0.00±0.00	0.22±0.06
$1/4^{th}$ of LC_{50}	15-day	1.00±0.03	0.36±0.03	0.29±0.04	1.65±0.04
	30-day	1.49±0.03	0.50±0.04	0.43±0.03	2.42±0.08
	45-day	0.68±0.04	0.11±0.04	0.06±0.03	0.85±0.05
	60-day	0.39±0.04	0.03±0.02	0.01±0.02	0.43±0.04
$1/3^{rd}$ of LC_{50}	15-day	1.25±0.03	0.53±0.02	0.40±0.03	2.18±0.04
	30-day	1.78±0.04	0.77±0.05	0.62±0.08	3.17±0.05
	45-day	0.89±0.04	0.27±0.03	0.13±0.05	1.29±0.03
	60-day	0.58±0.06	0.08±0.04	0.06±0.03	0.72±0.06

blood cells and our results showed the same genetic damage in term of micronucleus.

Different researchers as Muranli and Guner [45] Sarabia et al [46] showed that not only pyrethroids like Cypermethrin cause DNA damage but other pesticides like chlorpyrifos (organophosphate) do cause the same damage. morphological alterations which have been reported due to the treatment of pesticides (pyrethroids) in erythrocytes are binucleated erythrocytes lobed or notched nuclei along with blebbed membrane nuclei and micronuclei .These morphological changes could be the result of oxidative damage to mitochondrion. This oxidative damage also pledges the apoptotic changes like production of fodrin proteins and cleavage of cytoskeleton gelsolin and increased caspase activated DNase (CAD) in the nucleus which is responsible for the degradation, breakdown and disintegration of nuclear lamins proteins [47].

According to Çavaş and Gozukara [48] it is alluring to speculate that blebbed, notched and lobed nuclei could result from aneuploidy, i.e., a process leading to formation of chromosomal abnormalities and our results showed that DNA damage determined in-terms of percentage of damaged cells, micronuclei frequency and frequency of other nuclear abnormalities varied significantly due to exposure of various concentrations of pesticides mixtures. An exposure of $1/3^{rd}$ of LC_{50} to *C. idella* induced significantly higher number of other nuclear abnormalities in-terms of dumble shaped blebbed and notched nuclei. The total frequency of other nuclear abnormalities in peripheral erythrocytes of *C. idella*, exposed to various concentrations of pesticides mixture, was significantly higher at $1/3^{rd}$ of LC_{50} followed by that of positive and negative control.

4. CONCLUSION

It is concluded that pesticides mixture may cause biochemical disturbances and damage at molecular level to the tissues like kidney, liver, gills, brain, heart and muscles of fish. Therefore, claims of being lesser toxic to the untargeted animals may be true in limits of doses used as at sub-lethal level (LC_{50})

Pesticides exposure decreased one of the major antioxidant enzymes (Catalase) activities as they are considered as the first defense line of immune system. Moreover, these toxicants caused DNA damage (genotoxicity) at cellular level in terms of blebbed, notched, dumble shaped abnormalities in nuclei pesticides exposed *C. idella*.

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