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RESEARCH PAPER

Assessment of Antioxidant Biomarkers and Protein Levels in Tissues of *Oreochromis mossambicus* and *Channa punctatus* Exposed to Toxicity by Fungicides

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Abstract

The present investigation is about change in antioxidant enzymes and protein profile in *Oreochromis mossambicus* and *Channa punctatus* due to toxicity induced by application of fungicides in paddy-cum-fish ecosystem in India. Antioxidant enzymes like Lactate dehydrogenase (LDH), Malate dehydrogenase (MDH) and Peroxidase (Pox) are used as biomarkers for toxicological study. *Oreochromis mossambicus* and *Channa punctatus* were exposed to different concentration of Azoxystrobin 23% SC and Hexaconazole 5% SC respectively and LC_{50} was determined. LC_{50} for Amister was detected to be 0.008 ml/lit and that for Contaf plus was found to be 0.025 ml/lit. In case of *Oreochromis mossambicus* 1/2.5th, 1/5th and 1/7.5th of LC_{50} (0.0032 ml/lit, 0.0016 ml/lit and 0.001 ml/lit) were selected for chronic sub-lethal study. For *Channa punctatus* ½nd, ¼th and 1/6th of LC_{50} value (0.0125 ml/lit, 0.0062 ml/lit and 0.0041 ml/lit) were chosen. After 3 months of exposure, the individuals exposed to toxicity were sacrificed and stress enzymes and protein profile were checked in gill, heart, liver, kidney, muscle and spleen. Overall result revealed a gradual increase in LDH and Peroxidase but decrement in level of MDH and protein profile was also changed due to toxicity by fungicides.

Keywords: *Oreochromis mossambicus*, Channa punctatus, fungicides, Antioxidant enzymes, Protein profile.

Introduction

Stressor factors including water pollution, viral and bacterial infections, parasitic invasions, malnutrition, severe bleeding may induce to the perturbation of many haematochemical parameters in fish (Fazio, Marafioti, Arfuso, Ficcione, & Faggio, 2013, Fazio *et al*., 2014, Fazio, Piccone, Arfuso, & Faggio, 2015). Several researches have been carried out with the characterization of tissue and organ specific isoenzyme patterns (Mo *et al*., 1975; Seimiya *et al*., 1997) among which little were concerned with fishes. Few studies have concerned with the isoenzymatic profiles in the Nile tilapia (Li & Zhao, 2001; Shahjahan *et al*., 2008). Chaudhuri and Krishna (1998) reported the tissue specificity and variation in the degree of expression of five enzyme systems in *Labeo rohita* from river Yamuna in liver, muscle, heart and brain tissues. Certain antioxidant enzymes like LDH (lactate dehydrogenase), MDH (malate dehydrogenase), ALT (Alanine transaminase), AST (Aspartate transaminase), SDH (succinic dehydrogenase), and Peroxidase are being extensively used as potential biomarkers for measurement of tissue and organ damage due to pesticidal toxicity. LDH and MDH isoenzymes are major stress related enzyme systemfound in fishes.

As evidenced from previous studies, LDH is an important glycolytic enzyme present in all cells of almost all body tissues and changes in the enzyme activity may provide direct or indirect role to indicate the toxicity. Amacher (2002) said LDH is a terminal enzyme of anaerobic glycolysis and it mediates inter-converson of lactate to pyruvate depending on the availability of co-enzyme NAD. Therefore, being of crucial importance for muscular physiology, where under chemical stress more amount of energy is needed in short span of time (Coppo, Mussart, & Fioranelli, 2002; Baghi, Hassoun, & Stohs, 1995). The significant changes to enzyme activity indicates damage to any or all organs like liver, kidney or muscle injuries (Young, Dowman, & Cowell, 1999; De Coen, Jansen, & Segner, 2001).

Exposure to fungicides and insecticides causes decline in MDH and elevation in Peroxidase in all fish species. This enzyme not only converts malate to oxaloacetate but also plays a significant role in CO_2 fixation besides gluconeogenesis (Lehninger

principles of biochemistry 5th edition).

$$\text{Malate} + \text{NAD+} \rightarrow \text{Oxaloacetate} + \text{NADH}^{+} + \text{H}^{+}$$

Peroxidase enzyme is involved in phagocytosis and immune cell function (Rodriguez, Esteban, & Meseguer, 2003; Soares-da-Silva *et al*., 2002), cell adhesion (Holmblad & Soderhall, 1999), antioxidant function (Gamble, Goldfarb, Porte, & Livingstone, 1995; Galloway & Depledge, 2001) and helps in formation of melanin by oxidative polymerization of hydroquinones (D'Ischa, Napolitano, & Prota, 1991). Previous investigations confirmed that due to pesticidal impact on fish reveals changes in protein profile. It is also found that a high proteolytic activity or increased production of protease enzyme causes low levels of protein content in tissues of fish under stress (Baise, & Lokhande, 2012; Nagaraju, & Venkata, 2013). In this investigation, a comparative study for antioxidant biomarkers and protein profile has been presented due to toxicity induced by Amister (Azoxystrobin 23% SC) in *Oreochromis mossambicus* and Contaf plus (Hexaconazole 5% SC) applied to *Channa punctatus*.

Materials and Methods

Sample Collection and Acclimatization

The samples were collected from a local market of length 30±5 cm and weight 52±5 g. Prior to the experimentation the normal uninfected healthy fish were selected for experiment. The fish were cleaned under running tap water and disinfected using 0.02% KMNO4 and 0.004% formalin solution to remove external infection of fungi, algae, etc. The samples were acclimatized for 15 days to laboratory conditions and kept in aquariums containing 35 litre of water and regularly fed. The changes in physiochemical characteristics of water, such as temperature, pH, hardness, total alkalinity and DO (dissolved oxygen) of experimental water were recorded throughout the experimental period (Table 1).

Experimental Design

10 samples of each *Oreochromis mossambicus* and *Channa punctatus* were randomly selected from the stock and were exposed to ten different concentrations of fungicides Amister and Contaf plus respectively for 96 hr to determine mean lethal concentration (LC_{50}). Different concentrations of Amister (Azoxystrobin 23% SC) application was restricted from 0.001-0.010 ml/L (0.001, 0.002, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009, 0.01 ml/L) and Contaf plus (Hexaconazole 5% SC) were ranged from 0.005-0.05 ml/L (0.005, 0.01, 0.015, 0.02, 0.025, 0.03, 0.035, 0.04, 0.045, 0.05 ml/L). LC_{50} for Amister was detected to be 0.008 ml/L and that for Contaf plus was found to be 0.025 ml/L. In case of *Oreochromis mossambicus* 1/2.5th, 1/5th and 1/7.5th of LC_{50} (0.0032 ml/L, 0.0016 ml/L and 0.001 ml/L) were selected for chronic sub-lethal study. For *Channa punctatus* ½nd, ¼th and 1/6th of LC_{50} value (0.0125 ml/L, 0.0062 ml/L and 0.0041 ml/L) were chosen. A control group was maintained simultaneously in both the experiments. All these experiments were performed in triplicates.

The toxicated fish samples were subjected to Native PAGE for stress related enzymatic study and also protein profile was checked.

Native PAGE

At the end of 90 days, the toxicated fish samples were collected and subjected for enzymological study. For sample preparation 100 mg of different organs, gill, heart, liver, kidney, muscle and spleen were taken and menced in 500 µl (1:5) of 0.1 M Tris-HCl buffer (pH 7.4). The homogenized lysate was centrifuged at 14000 rpm for 40 min at 4°C and supernatent was preserved for enzyme analysis (Native PAGE). Antioxidant enzymatic study for LDH, MDH and Peroxidase was done. Native PAGE (10% native polyacrylamide gel) was performed for qualitative study of LDH, MDH and Peroxidase as described by Stegmann *et al*., (1985) for different tissues, gill, heart, liver, kidney and spleen.

Gel Staining

The staining procedure was followed as described by Bader (1998).

LDH: After electrophoresis, the gel was incubated in 100 ml of 0.05 M Tris HCl pH 8.5 containing 25 mg NBT, 25 mg EDTA, 25 mg NAD, 1 ml lactic acid and 3 mg PMS and kept for 15-30 min in dark.

MDH: After electrophoresis, the native gel was placed in 0.05 M Tris HCl pH 8.5 (100 ml) containing 25 mg NBT, 25 mg EDTA, 25 mg NAD, 10 mg malic acid and 3 mg PMS for 15-30 min in dark.

Peroxidase: After gel run, incubation was done

Table 1. Hydrographical condition of control tank and testing tank

Parameters	Concentrations	
	Control tank	Test tank
Temperature (°C)	27±2	29±2
pH	7.4	8.1
Dissolved oxygen (mg/L)	8.0	3.8
Hardness (mg/L)	116	864
Total alkinity (mg/L)	27	118

in 100 ml of Tris-HCl buffer bearing 50 mg O-dianisidine (previously dissolved in few drops of acetic acid) and 1 ml of hydrogen peroxide and left for 15-30 min in dark.

Isolation of Protein

500 mg fish organs (gill, heart, liver, kidney, muscle and spleen) were thawed in urea-thiourea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 45 mM Tris, 60 mM DTT and protease inhibitor- PMSF). Thawed samples were vortexed and kept at 4°C for 30 min. Mechanically disrupted and kept on ice. Samples were adjusted to 900 µl of lysis buffer (20 mM Tris, 100 mM NaCl, 1% Triton and Protease inhibitor - PMSF) and incubated for 15 min at 35°C. Reincubated in ice for 10 min. 100 µl of lysis buffer was added and incubated for 10 min along with DNAse I. The samples were centrifuged at 12000 rpm for 15 min at 4°C (middle aq phase bears protein). Extended delipidation was accompanied by tri-n-butylphophate-acetone-methanol precipitation. Precipitated proteins were estimated subjected to SDS-PAGE.

Protein Estimation

Protein concentration was calculated by Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951). Absorbance was measured at 750 nm by UV-VIS Elico spectrophotometer.

SDS-PAGE of Protein Profile

SDS-PAGE was performed for protein profiling of fungicides exposed fishes. 10% polyacrylamide gel was casted according to Stegmann, Hoekstra, Scherphof, and Wilschut (1985) and with Coomasie Brilliant Blue by incubating it overnight in staining solution bearing the dye followed by destaining (Methanol: 25 ml, Glacial acetic acid: 25 ml and water: 200 ml) and photographed.

Data Analysis

LC_{50} was determined using SPSS Vs. 17 and enzymological calculations were done by Student's t-Test.

Results

Native PAGE of Stress Enzymes in *Oreochromis mossambicus*

The results are summarized in Table 2, 3 and 4. The gradual increase of lactate dehydrogenase (LDH) and peroxidase (Pox) in gill, heart, kidney, liver, muscle and spleen exposure to 30 d, 60 d and 90 d for 0.001, 0.0016 and 0.0032 ml/L. The sub-lethal concentrations of Azoxystrobin caused significant depletion *(*$P<0.05$*)* of malate dehydrogenase (MDH), which was more pronounced at 60 d and 90 d exposure of the organism. The elevation of LDH in gill was observed 7.89% (30 d) to 44.73% (90 d) for

Table 2. Activities of LDH, MDH and Pox in *Oreochromis mossambicus* exposed to Azoxystrobin 23% SC, Dose1: 0.001 ml/L, ($P<0.05$)

Organs	No. of fish	Control	Toxicated		
			30d	60d	90d
Gill	10				
LDH		0.385±0.12	0.412±0.16 (7.89)	0.466±0.17 (21.05)	0.559±0.12 (44.73)
MDH		0.387±0.11	0.34±0.15 (-10.52)	0.321±0.16 (-15.78)	0.303±0.14 (-21.05)
Peroxidase		0.478±0.11	0.481±0.13 (2.12)	0.499±0.14 (4.25)	0.526±0.18 (10.63)
Heart	10				
LDH		0.277±0.11	0.281±0.12 (3.7)	0.322±0.12 (18.51)	0.342±0.13 (25.92)
MDH		0.284±0.11	0.263±0.12 (-7.14)	0.24±0.18 (-14.28)	0.22±0.17 (-21.42)
Peroxidase		0.498±0.23	0.502±0.17 (2.04)	0.517±0.16 (4.08)	0.539±0.18 (8.16)
Liver	10				
LDH		0.496±0.22	0.526±0.18 (6.12)	0.64±0.20 (30.61)	0.684±0.21 (38.77)
MDH		0.52±0.20	0.50±0.22 (-3.84)	0.478±0.20 (-9.61)	0.45±0.13 (-13.46)
Peroxidase		0.597±0.35	0.619±0.29 (3.39)	0.628±0.16 (5.08)	0.682±0.15 (15.25)
Kidney	10				
LDH		0.331±0.18	0.356±0.15 (6.06)	0.367±0.16 (9.09)	0.412±0.21 (24.24)
MDH		0.341±0.22	0.336±0.19 (-2.94)	0.329±0.17 (-5.88)	0.314±0.13 (-8.82)
Peroxidase		0.554±0.16	0.57±0.18 (3.63)	0.603±0.20 (9.09)	0.628±0.18 (12.72)
Muscle	10				
LDH		0.354±0.14	0.409±0.13 (14.28)	0.458±0.22 (28.57)	0.537±0.12 (51.42)
MDH		0.48±0.15	0.462±0.14 (-4.16)	0.429±0.12 (-12.5)	0.433±0.16 (-10.41)
Peroxidase		0.426±0.14	0.437±0.19 (2.38)	0.452±0.15 (7.14)	0.477±0.19 (11.9)
Spleen	10				
LDH		0.283±0.13	0.326±0.13 (14.28)	0.344±0.12 (21.42)	0.398±0.11 (39.28)
MDH		0.297±0.14	0.276±0.11 (-6.89)	0.262±0.15 (-10.34)	0.235±0.13 (-20.69)
Peroxidase		0.366±0.11	0.389±0.15 (5.55)	0.396±0.11 (8.33)	0.412±0.16 (13.88)

Table 3. Activities of LDH, MDH and Pox in *Oreochromis mossambicus* exposed to Azoxystrobin 23 % SC, Dose2: 0.0016 ml/L, *(*P<0.05*)*

Organs	No. of fish	Control	Toxicated		
			30d	60d	90d
Gill	10				
LDH		0.593±0.18	0.598±0.16 (0.84)	0.662±0.17 (11.29)	0.704±0.12 (18.04)
MDH		0.497±0.15	0.45±0.23 (-8.16)	0.38±0.18 (-22.44)	0.28±0.13 (-42.85)
Peroxidase		0.405±0.11	0.56±0.13 (40)	0.614±0.11 (52.5)	0.689±0.16 (70)
Heart	10				
LDH		0.32±0.11	0.35±0.12 (9.37)	0.39±0.12 (21.87)	0.43±0.13 (34.37)
MDH		0.308±0.11	0.258±0.14 (-18.83)	0.22±0.10 (-28.57)	0.18±0.09 (-41.55)
Peroxidase		0.538±0.20	0.55±0.19 (3.77)	0.582±0.15 (9.43)	0.593±0.17 (11.32)
Liver	10				
LDH		0.626±0.12	0.714±0.28 (14.51)	0.84±0.19 (35.48)	0.904±0.16 (45.16)
MDH		0.72±0.17	0.45±0.23 (-37.5)	0.438±0.20 (-40.27)	0.405±0.18 (-43.75)
Peroxidase		0.53±0.20	0.694±0.19 (30.18)	0.734±0.16 (37.73)	0.823±0.24 (54.71)
Kidney	10				
LDH		0.317±0.18	0.381±0.15 (22.58)	0.438±0.16 (38.71)	0.602±0.11 (94.19)
MDH		0.362±0.12	0.39±0.13 (-8.33)	0.318±0.19 (-13.88)	0.28±0.12 (-22.22)
Peroxidase		0.454±0.16	0.65±0.12 (44.44)	0.717±0.13 (57.77)	0.78±0.13 (73.33)
Muscle	10				
LDH		0.487±0.14	0.535±0.13 (10.41)	0.579±0.22 (18.75)	0.634±0.12 (31.25)
MDH		0.53±0.15	0.43±0.12 (-18.86)	0.40±0.11 (-24.52)	0.396±0.19 (-26.41)
Peroxidase		0.39±0.14	0.479±0.16 (20.51)	0.482±0.18 (23.07)	0.63±0.19 (61.53)
Spleen	10				
LDH		0.304±0.17	0.326±0.13 (6.66)	0.38±0.12 (26.66)	0.445±0.21 (46.66)
MDH		0.357±0.24	0.305±0.18 (-14.28)	0.278±0.19 (-22.85)	0.22±0.15 (-37.14)
Peroxidase		0.426±0.11	0.513±0.15 (21.42)	0.56±0.19 (33.33)	0.623±0.16 (47.61)

Table 4. Activities of LDH, MDH and Pox in *Oreochromis mossambicus* exposed to Azoxystrobin 23% SC, Dose3: 0.0032 ml/L., (P<0.05)

Organs	No. of fish	Control	Toxicated		
			30d	60d	90d
Gill	10				
LDH		0.427±0.21	0.73±0.16 (73.81)	0.862±0.17 (104.76)	0.902±0.12 (114.28)
MDH		0.497±0.11	0.25±0.13 (-48.98)	0.23±0.18 (-53.06)	0.218±0.13 (-57.14)
Peroxidase		0.395±0.11	0.76±0.12 (94.87)	0.86±0.11 (120.51)	0.91±0.18 (133.33)
Heart	10				
LDH		0.32±0.11	0.45±0.12 (40.62)	0.58±0.12 (81.25)	0.63±0.16 (96.87)
MDH		0.28±0.19	0.18±0.10 (-35.71)	0.173±0.08 (-39.28)	0.16±0.09 (-42.85)
Peroxidase		0.538±0.13	0.67±0.19 (26.41)	0.662±0.15 (24.52)	0.73±0.17 (37.73)
Liver	10				
LDH		0.58±0.22	0.97±0.23 (67.24)	1.02±0.19 (75.86)	1.24±0.23 (113.79)
MDH		0.72±0.17	0.406±0.13 (-43.61)	0.38±0.15 (-47.22)	0.35±0.18 (-51.38)
Peroxidase		0.53±0.15	0.94±0.19 (77.35)	1.14±0.21 (115.09)	1.23±0.21 (132.07)
Kidney	10				
LDH		0.34±0.20	0.61±0.23 (79.41)	0.68±0.21 (100)	0.72±0.23 (111.76)
MDH		0.32±0.20	0.24±0.20 (-25)	0.21±0.19 (-34.37)	0.19±0.12 (-40.62)
Peroxidase		0.454±0.16	0.81±0.12 (80)	0.875±0.13 (93.33)	0.92±0.16 (104.44)
Muscle	10				
LDH		0.487±0.14	0.75±0.13 (56.25)	0.77±0.22 (60.41)	0.812±0.19 (68.75)
MDH		0.53±0.15	0.42±0.12 (-20.75)	0.38±0.11 (-28.3)	0.33±0.12 (-37.73)
Peroxidase		0.42±0.14	0.72±0.16 (71.42)	0.79±0.18 (88.09)	0.82±0.19 (95.23)
Spleen	10				
LDH		0.304±0.17	0.46±0.13 (53.33)	0.64±0.12 (113.33)	0.75±0.21 (150)
MDH		0.47±0.14	0.215±0.18 (-55.31)	0.18±0.12 (-61.7)	0.17±0.15 (-63.83)
Peroxidase		0.426±0.11	0.63±0.15 (50)	0.66±0.15 (57.14)	0.73±0.16 (73.81)

0.001 ml/L which is much less than 0.0032 ml/L. At acute sub-lethal dose (0.0032 ml/L) it was recorded as 73.81% (30 d) to 114.28% (90 d). The depletion of malate dehydrogenase (MDH) varies in different tissues at all concentration of Amister. The decline in this enzyme was observed least in heart (-42.85%) at

90 d exposure under 0.0032 ml/L whereas it was noticed -63.83% in spleen followed by gill (-57.14%) and liver (-51.38%). The escalation of peroxidase was observed in gill at 0.0032 ml/L for 90 d, which ranged from (94.87-133.33)% followed by liver (77.35-132.07)% whereas in muscle it was recorded 95.23% at 90 d exposure of same chemical.

The different stress related antioxidant enzymes in the toxicated fish (exposed for 90 d under Azoxystrobin) subjected for Native PAGE were compiled in Figure 1a and 1b (LDH), Figure 2a and 2b (MDH) and Figure 3a and 3b (Pox). Lactate dehydrogenase showed six isomorphic bands (LDH 1-6). All the isomorphs were expressed in liver and muscle, but LDH 6 was weakly expressed in both the organs. LDH 1, 2 and 3 were absent in spleen and heart but LDH 6 was intensely expressed in heart. In case of the stress enzyme peroxidase, six isomorphic

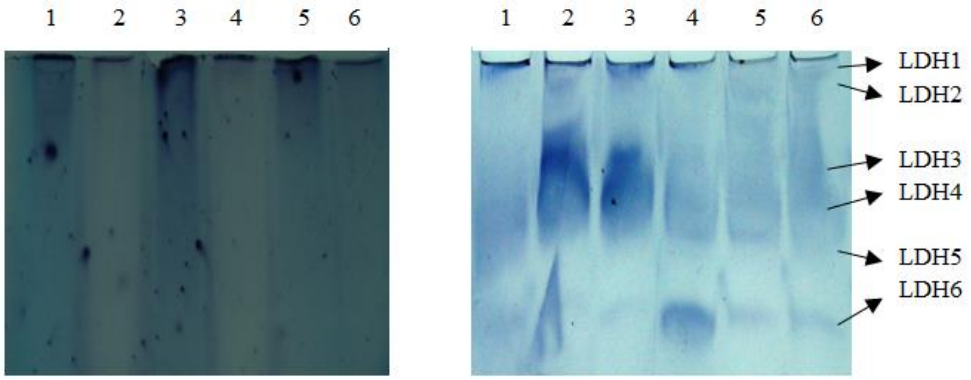

Figure 1. A. Control: LDH: 1-6: Gill, Heart, Liver, Muscle, Kidney, Spleen (No bands visible); B. Toxicated: LDH 1-6: Spleen, Liver, Muscle, Heart, Kidney, Gill.

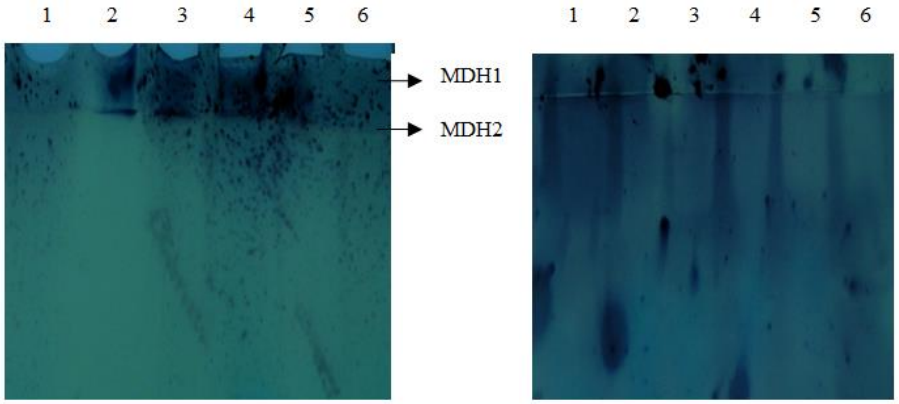

Figure 2. A. Control: MDH: 1-6: Gill, Heart, Liver, Muscle, Kidney, Spleen; B. Toxicated: MDH 1-6: Spleen, Liver, Muscle, Heart, Kidney, Gill (No bands visible).

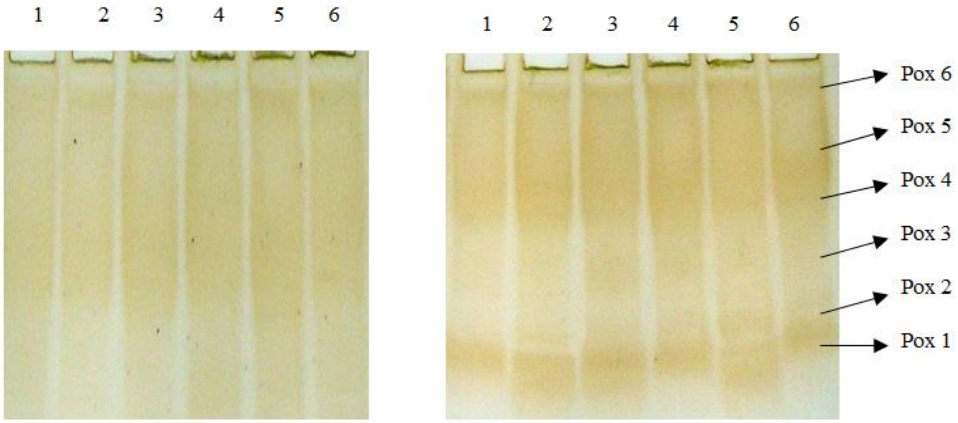

Figure 3. A. Control: Pox: 1-6: Gill, Heart, Liver, Muscle, Kidney, Spleen (No bands visible); B. Toxicated: Pox: 1-6: Gill, Liver, Muscle, Heart, Kidney, Spleen.

bands (Pox 1-6) were intensely appeared as tissue specific manner under acute stress (0.0032 ml/L of Amister for 90 d in liver, muscle and kidney followed by heart, spleen and gill (Pox 6 was absent). Native-PAGE showed a sharp elevation of Lactate dehydrogenase and Peroxidase and a decline of Malate dehydrogenase in *Oreochromis mossambicus* due to exposure of Amister 23 %SC.

Protein Profile in *Oreochromis mossambicus*

The results revealed that long exposure of *O. mossambicus* to pesticides interferes with protein metabolism. Total protein content was drastically reduced in all organs due to exposure of Amister 23 % SC after 90 d. Few intense bands were appeared with the expected size of ~51, ~62 and ~70 kDa in control samples and treated fish showed ~42 kDa additional protein band in all tissues. Lower molecular weight proteins appeared in treated fish as evidenced in SDS-PAGE (Figure 4a and 4b).

Native PAGE of Stress Enzymes in *Channa punctatus*

There was gradual increase for lactate dehydrogenase (LDH) and peroxidase (Pox) in gill, heart, kidney, liver, muscle and spleen for all three exposure periods (30 d, 60 d and 90 d) for 0.0041, 0.0062 and 0.0125 ml/L (Tables 5, 6, 7). The sub-lethal concentrations of hexaconazole caused significant depletion *(*P<0.05*)* for malate dehydrogenase (MDH) at 60 d and 90 d exposure of the organism. Increase of lactate dehydrogenase was found in gill (0.387±0.16 to 0.48±0.82), in heart (0.25±0.82 to 0.301±0.63), in liver (0.456±0.28 to 0.61±0.36), in kidney (0.31±0.45 to 0.35±0.31), in muscle (0.29±0.13 to 0.417±0.12) and in spleen (0.29±0.63 to 0.324±0.21) upon exposure of Hexaconazole 5 %SC. Similarly significant increase of LDH was recorded at 0.0125 ml/L which was 0.62±0.16 to 0.68±0.82 in gill, 0.45±0.82 to 0.53±0.63 in heart, 0.75±0.28 to 0.83±0.36 in liver, 0.56±0.45 to 0.64±0.31 in kidney, 0.55±0.13 to 0.71±0.12 in muscle and 0.41±0.63 to 0.44±0.21 in spleen of *Channa punctatus* respectively in compared to untreated control fish.

The depletion of malate dehydrogenase (MDH) varied greatly in the organs at all concentration of Hexaconazole 5 %SC. The MDH was significantly reduced *(*P<0.05*)* in kidney (-37.66%) followed by gill (-36.11%) and heart (-32.25%) and muscle (-20.83%) over control under chronic exposure to hexaconazole 5 %SC at 0.0125 ml/L. The range of decline in MDH in different organs under lowest sub-lethal dose from 30 d to 90 d was observed in gill (0.34±0.35 to 0.31±0.74), heart (0.28±0.32 to 0.26±0.17), liver (0.423±0.22 to 0.42±0.13), kidney (0.36±0.61 to 0.31±0.93 to), muscle (0.46±0.34 to 0.42±0.56) and in spleen (0.28±0.11 to 0.24±0.73).

As the results indicated there is a gradual increment in antioxidant enzyme peroxidase was noticed in all organs for all concentrations of fungicides. The percentage elevation over control of peroxidase was highest in gill (61.53-84.61%) followed by spleen (52.77-63.88%), muscle (14.28-52.38%) and heart (37.77-51.11%) under 0.0125 ml/L of Hexaconazole 5% SC for 30-90 d. The percent increase of Pox was very high during exposure of Hexaconazole 5% SC at 0.0125 ml/L at 30-90 d which ranged from 0.45±0.13 to 0.51±0.28 in gill, 0.48±0.17 to 0.52±0.48 in heart, 0.55±0.21 to 0.58±0.35 in liver, 0.57±0.68 to 0.62±0.38 in kidney, 0.43±0.29 to 0.46±0.19 in muscle and 0.41±0.55 to 0.44±0.52 in spleen respectively.

There was a sharp elevation of Lactate dehydrogenase and Peroxidase and a decline was noticed for Malate dehydrogenase. Lactate dehydrogenase showed two isomorphic bands (LDH 1-2) which were abolished in control. LDH 1 was significantly expressed in all organs but LDH 2 was visible only in heart of toxicated fish. LDH 1 was intensely expressed in spleen, kidney, muscle and liver as compared to other organs. For MDH three

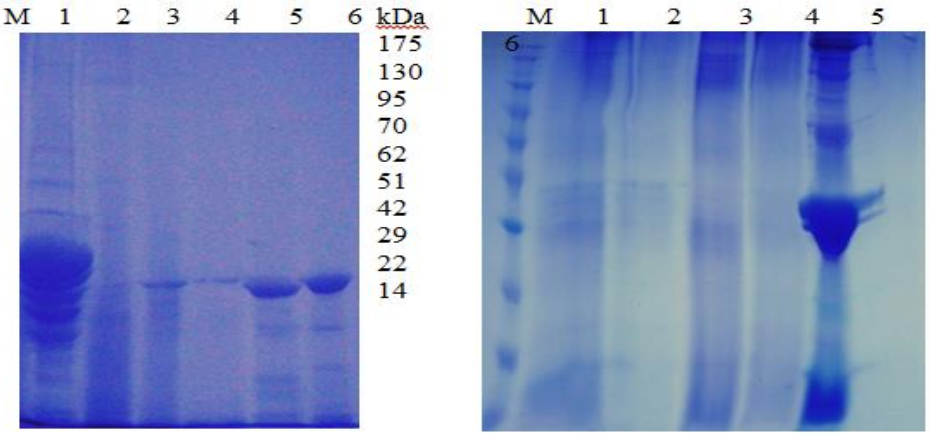

Figure 4. A. Toxicated: 1-6: Muscle, gill, heart, liver kidney, spleen. B. Control, 1-6: gill, heart, liver, kidney, muscle, spleen.

Table 5. Activities of LDH, MDH and Pox in *Channa punctatus* exposed to Hexaconazole 5% SC, Dose1: 0.0041 ml/L

Organs	No. of fish	Control	Toxicated		
			30d	60d	90d
Gill	10				
LDH		0.35±0.18	0.387±0.16 (10.57)	0.42±0.17 (20)	0.48±0.12 (37.14)
MDH		0.36±0.11	0.34±0.15 (-5.55)	0.321±0.16 (-10.83)	0.31±0.14 (-13.88)
Peroxidase		0.393±0.11	0.45±0.13 (14.5)	0.49±0.24 (24.68)	0.51±0.28 (29.77)
Heart	10				
LDH		0.22±0.11	0.25±0.12 (13.63)	0.282±0.12 (28.18)	0.301±0.13 (36.81)
MDH		0.31±0.10	0.28±0.12 (-9.67)	0.276±0.18 (-10.96)	0.268±0.17 (-13.54)
Peroxidase		0.45±0.23	0.482±0.17 (7.11)	0.497±0.16 (10.44)	0.52±0.18 (15.55)
Liver	10				
LDH		0.42±0.22	0.456±0.20 (8.57)	0.54±0.20 (28.57)	0.61±0.23 (45.23)
MDH		0.43±0.21	0.423±0.20 (-1.62)	0.47±0.22 (-9.3)	0.42±0.19 (-2.32)
Peroxidase		0.52±0.15	0.55±0.21 (5.76)	0.56±0.26 (7.69)	0.58±0.25 (11.53)
Kidney	10				
LDH		0.306±0.18	0.31±0.15 (1.3)	0.33±0.16 (7.84)	0.355±0.11 (16.01)
MDH		0.369±0.22	0.36±0.19 (-2.43)	0.329±0.17 (-10.84)	0.314±0.13 (-14.9)
Peroxidase		0.55±0.16	0.57±0.18 (3.63)	0.603±0.10 (9.63)	0.628±0.18 (14.18)
Muscle	10				
LDH		0.25±0.14	0.29±0.13 (16)	0.33±0.20 (32)	0.417±0.12 (66.8)
MDH		0.48±0.15	0.46±0.14 (-4.16)	0.44±0.12 (-8.33)	0.426±0.16 (-11.25)
Peroxidase		0.426±0.14	0.435±0.20 (2.11)	0.442±0.15 (3.75)	0.46±0.19 (7.98)
Spleen	10				
LDH		0.283±0.13	0.29±0.13 (2.47)	0.32±0.12 (13.07)	0.324±0.10 (14.48)
MDH		0.297±0.14	0.283±0.11 (-4.71)	0.26±0.15 (-12.45)	0.24±0.13 (-19.19)
Peroxidase		0.366±0.11	0.414±0.15 (13.11)	0.42±0.12 (14.75)	0.44±0.12 (20.21)

Values expressed in Mean±S.D. of 10 replicates. Student t-Test was performed between control and exposed values. The mean values were found to be significantly different at 5% level of signicance *(*P<0.05*)*. Percentage increase or decrease over control were expressed within brackets

Table 6. Activities of LDH, MDH and Pox in *Channa punctatus* exposed to Hexaconazole 5% SC, Dose2: 0.0062 ml/L., (P <0.05)

Organs	No. of fish	Control	Toxicated		
			30d	60d	90d
Gill	10				
LDH		0.35±0.19	0.48±0.16 (37.14)	0.52±0.17 (48.57)	0.58±0.12 (65.71)
MDH		0.36±0.11	0.34±0.15 (-5.55)	0.33±0.16 (-8.33)	0.30±0.14 (-16.66)
Peroxidase		0.393±0.10	0.55±0.13 (41.02)	0.59±0.14 (51.28)	0.61±0.18 (56.41)
Heart	10				
LDH		0.32±0.13	0.435±0.12 (34.37)	0.452±0.12 (40.62)	0.48±0.13 (50)
MDH		0.31±0.11	0.26±0.12 (-16.12)	0.256±0.18 (-19.35)	0.24±0.17 (-22.58)
Peroxidase		0.45±0.23	0.58±0.17 (28.88)	0.597±0.16 (31.11)	0.62±0.18 (37.77)
Liver	10				
LDH		0.42±0.12	0.65±0.10 (54.76)	0.69±0.11 (64.28)	0.71±0.16 (69.04)
MDH		0.43±0.17	0.40±0.22 (-6.97)	0.37±0.20 (-13.95)	0.35±0.13 (-18.6)
Peroxidase		0.52±0.15	0.58±0.21 (11.53)	0.59±0.16 (13.46)	0.66±0.15 (26.92)
Kidney	10				
LDH		0.306±0.18	0.44±0.15 (43.79)	0.46±0.16 (50.32)	0.50±0.11 (63.39)
MDH		0.369±0.12	0.31±0.11 (-15.98)	0.29±0.17 (-21.4)	0.28±0.13 (-24.11)
Peroxidase		0.55±0.16	0.57±0.18 (3.63)	0.61±0.21 (10.9)	0.62±0.18 (12.72)
Muscle	10				
LDH		0.25±0.14	0.49±0.13 (96)	0.51±0.22 (104)	0.53±0.12 (112)
MDH		0.48±0.15	0.42±0.14 (-12.5)	0.41±0.12 (-14.58)	0.402±0.16 (-16.66)
Peroxidase		0.426±0.14	0.48±0.19 (14.28)	0.49±0.15 (16.66)	0.51±0.19 (21.42)
Spleen	10				
LDH		0.283±0.13	0.42±0.13 (50)	0.43±0.12 (53.57)	0.44±0.11 (57.14)
MDH		0.297±0.14	0.243±0.10 (-17.24)	0.22±0.12 (-24.13)	0.21±0.13 (-27.58)
Peroxidase		0.366±0.16	0.45±0.15 (25)	0.46±0.11 (27.77)	0.48±0.12 (44.44)

Table 7. Activities of LDH, MDH and Pox in *Channa punctatus* exposed to Hexaconazole 5% SC, Dose3: 0.0125 ml/L., (P <0.05)

Organs	No. of fish	Control	Toxicated		
			30d	60d	90d
Gill	10				
LDH		0.35±0.19	0.62±0.16 (77.14)	0.64±0.17 (82.85)	0.68±0.12 (94.28)
MDH		0.36±0.15	0.31±0.15 (-13.88)	0.28±0.16 (-22.22)	0.23±0.14 (-36.11)
Peroxidase		0.393±0.11	0.63±0.13 (61.53)	0.67±0.24 (71.79)	0.72±0.28 (84.61)
Heart	10				
LDH		0.32±0.11	0.45±0.12 (40.62)	0.49±0.12 (53.12)	0.53±0.13 (65.62)
MDH		0.31±0.10	0.26±0.12 (-16.12)	0.22±0.18 (-29.03)	0.21±0.17 (-32.25)
Peroxidase		0.45±0.23	0.62±0.17 (37.77)	0.65±0.16 (44.44)	0.68±0.18 (51.11)
Liver	10				
LDH		0.42±0.12	0.75±0.20 (78.57)	0.79±0.19 (88.09)	0.83±0.16 (97.61)
MDH		0.43±0.20	0.36±0.17 (-16.27)	0.33±0.12 (-23.25)	0.32±0.13 (-25.58)
Peroxidase		0.52±0.15	0.67±0.21 (28.84)	0.69±0.16 (32.69)	0.76±0.15 (46.15)
Kidney	10				
LDH		0.306±0.18	0.56±0.15 (83)	0.58±0.16 (89.54)	0.64±0.13 (109.15)
MDH		0.369±0.12	0.29±0.11 (-21.4)	0.26±0.17 (-29.53)	0.23±0.13 (-37.66)
Peroxidase		0.55±0.16	0.66±0.18 (20)	0.68±0.11 (23.63)	0.69±0.18 (25.45)
Muscle	10				
LDH		0.35±0.14	0.55±0.13 (57.14)	0.58±0.22 (65.71)	0.71±0.12 (102.85)
MDH		0.48±0.15	0.42±0.14 (-12.5)	0.41±0.12 (-14.58)	0.38±0.16 (-20.83)
Peroxidase		0.426±0.14	0.48±0.19 (14.28)	0.52±0.15 (23.81)	0.64±0.19 (52.38)
Spleen	10				
LDH		0.283±0.13	0.41±0.23 (46.42)	0.43±0.22 (53.57)	0.44±0.21 (57.14)
MDH		0.297±0.14	0.23±0.17 (-20.69)	0.22±0.15 (-24.13)	0.208±0.13 (-28.27)
Peroxidase		0.366±0.11	0.55±0.12 (52.77)	0.56±0.14 (55.55)	0.59±0.12 (63.88)

isomorphic bands (MDH 1, MDH 2 and MDH 3) appeared in control *Channa punctatus* which were not significantly expressed after 90 d of exposure under Hexaconazole 5% SC. The native PAGE revealed that all isomorphs of MDH were highly observed in gill and kidney followed by heart, liver, spleen and muscle. In case of the stress enzyme peroxidase, four isomorphic bands (Pox 1-4) were observed as tissue specific manner under acute stress (0.0125 ml/L. of Hexaconazole 5% SC for 90 d in *Channa punctatus*. The different antioxidant enzymes responding under stress in the fish (exposed for 90d under Hexaconazole 5 %SC) subjected for Native PAGE were compiled in Figure 5a and 5b (LDH), Figure 6a and 6b (MDH) and Figure 7a and 7b (Pox).

Protein Profile in *Channa punctatus*

The differential results of the SDS-PAGE of the protein profiling of the target organs of the fish exposure to Hexaconazole 5 %SC was observed. At 90 d of exposure, *Channa punctatus* showed over expression of some lower molecular weight protein which was ~10.5, ~14, ~22, ~29 and ~42 kDa. High molecular weight bands were observed in control which disappeared in toxicated fish. After 3 month of exposure lower molecular weight proteins get elevated as evidenced through gel picture. Figure 8a and 8b reveals gel image of SDS-PAGE of control and toxicated fish respectively.

Discussion

The present study on the impact of fungicides on fish species exposed to chemical toxicants revealed the significant change in antioxidant enzymes and protein profile. In presence of pollutants there is oxygen stress and organisms switch from aerobic to anaerobic metabolism to sustain and then antioxidant enzymes which constitute the defense system of organism come into play.

In the present study, it was observed that the activity of LDH was increased following sublethal exposures of fungicides azoxystrobin and hexaconazole in all the tissues of *Oreochromis mossambicus* and *Channa punctatus* throughout the experiment. Our result is in consistent with Martinez, Raynard, Bernard, and Chapman (2011), who worked on *Clarius batracus*. They reported that under hypoxic conditions, anaerobic glycolysis was activated. Simultaneously, at this stage, serum glucose as well as LDH activity in oxidative tissues, liver and gills, at 12 h at experimental hypoxia level, were found to be significantly increased. Consequently, we observed higher LDH activity in liver and gill tissues of toxicated fish. They also suggested that it may be due to clearance of blood lactate and the provision of glucose for metabolism by vital organs like heart and brain. Increased LDH resulted in lactic acidosis. Rees, Boily and Williamson (2009) documented that it is due to conversion of pyruvate to lactate. Similarly,

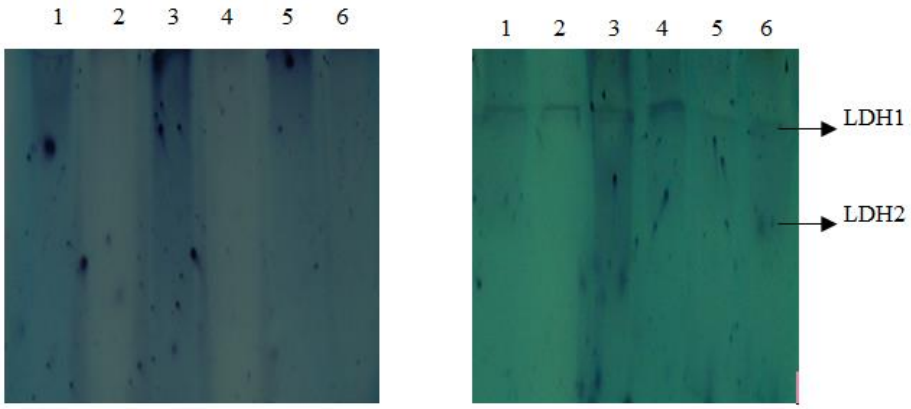

Figure 5. A. Control: LDH: 1-Gill, 2-heart, 3-liver, 4-muscle, 5-kidney, 6-spleen (No bands visible); B. Toxicated: LDH 1-spleen, 2- kidney, 3-muscle, 4-liver, 5-gill, 6-heart

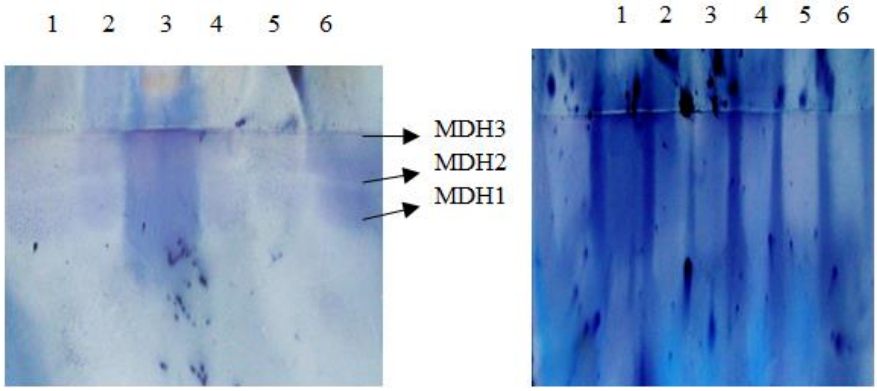

Figure 6. A. Control:MDH 1-liver, 2- heart, 3-kidney, 4-spleen, 5- muscle, 6-gill; B. Toxicated: MDH 1- liver, 2- kidney, 3- spleen, 4-muscle, 5- heart, 6- Gill (No bands visible).

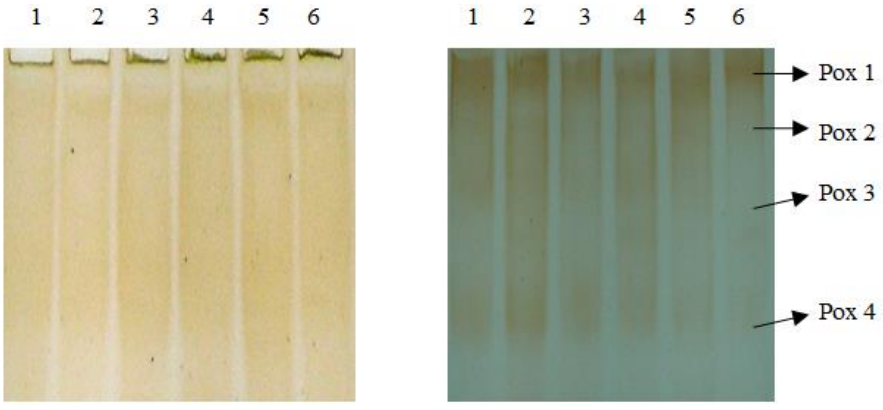

Figure 7. A. Control Peroxidase : 1-Gill, 2-heart, 3-liver, 4-muscle, 5-kidney, 6-spleen (No bands visible); B. Toxicated Peroxidase 1-muscle, 2-gill, 3-liver, 4-spleen, 5-heart, 6-kidney.

Sharma and Jain (2008) observed that under hypoxic condition there is augmented release of LDH into the tissues. Similar reports were given by Tiwari and Singh (2009). They observed decrease in MDH values in tissues of *Clarias batrachus* on exposure to endosulfan. A reduction in MDH activity was observed in matrix, *Brycon cephalus* after exposure to Folidol 600 (Archana & Gaikwad, 1998). Reduction of MDH indicates pesticides significantly inhibit aerobic respiration under stress (Tiwari, Pandey, & Singh, 2008). Elavation of peroxidase was also suggested by different studies which witness

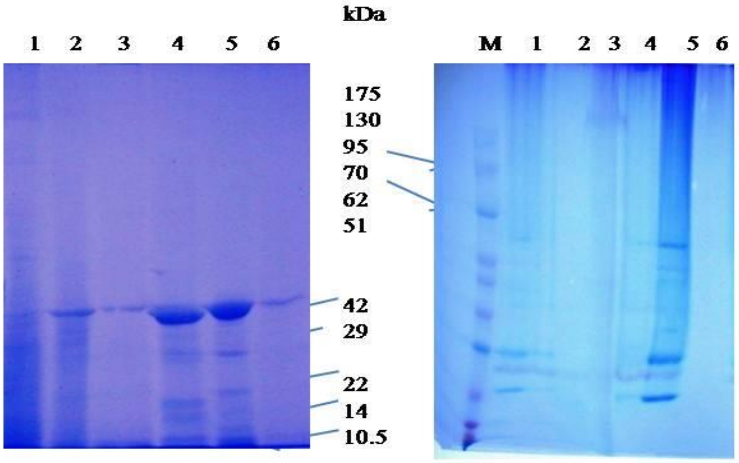

Figure 8. A. Control 1-6: gill, heart, liver, kidney, muscle, spleen. B. Toxicated 1-6: Muscle, gill, heart, liver kidney, spleen.

devastating effects of pesticides in diverse antioxidant enzyme activities (Ullah, R., Zuberi, Ullah, S., Ullah, I., & Dawar, 2014).

Impact of sub-lethal doses of fungicides (Amister and Hexaconazole) showed decline in protein content under chronic exposure to both fish species. It is well documented that pesticides alter the total protein content in different tissues of fish (Ahmad, 2012). Our results is in accordance with several previous researchers such as, Phenyl mercuric acetate (heavy metal) induced low protein level in muscles and liver of *Channa punctatus* (Karuppasamy, 2000). Cypermethrin exposure resulted in significant decrease in protein in endangered cyprinid fish *Tor putitora* (Ullah *et al*., 2014) and *Colisa fasciatus* (Singh & Singh, 2010). A pesticidal mixture used against *Clarias batrachus* induced changes in protein content (Jha & Verma, 2002). Bibi *et al*. (2014) proposed decreased protein contents in *Cyprinus carpio* due to karate. In another study, monocrotrophs declined lipid, protein and carbohydrate content in *Labeo rohita* (Muthukumaravel, Sivakumar, Kumarasamy, & Govindarajan, 2013).

Present investigation clearly shows that a high energy demand is the reason behind enhanced breakdown of proteins in blood thereby reducing the serum proteins' content. It is also found that a high proteolytic activity or increased production of protease enzyme or low protein genesis could cause decrement in protein content in tissues of fish under stress. David, Mushigeri, Sivakumar, & Philip (2004) Parthasarathy and Joseph (2011) also demonstrated the similar remarks in *Cyprinus carpio* and *Oreochromis mossambicus* exposed to cypermethrin and λ-cyhalothrin respectively.

Not only in animals but in plants also similar results were reported by Ganguly, Bhattacharya, Mandi and Tarafdar (2010). They stated induction of increased/decreased activity of particular isoform(s) of SOD, POD, and EST and expression of additional protein led to the apparent conclusion of linkage of alter metabolism of reactive electrophilic products due to exposure of insecticides in *Lathyrus sativus*.

The present study also indicated that the antioxidant enzymes LDH, MDH and Pox may be used as bio-chemical markers for eco-toxicity studies would be appropriate for hazard identification.

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