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Research Article

Evaluating the haematological and biochemical changes following acute toxicity of cadmium in air-breathing perch *Anabas testudineus* (Bloch, 1792)

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ABSTRACT

The climbing perch, *Anabas testudineus* (Bloch, 1792) is a potamodromous species and the adults usually inhabit rivers, flooded fields, and stagnant water bodies. Though, their natural populations are declining rapidly in their natural habitat due to aquatic pollution. Among the aquatic toxic pollutants, Cadmium (Cd) is considered as the most hazardous in the ecotoxicological aspect. Hence, the present work is designed to understand the physiological stress response leading to their cause of decline in natural habitat. For this, different hematological and biochemical parameters were analyzed in *A. testudineus* along with their behaviour upon acute Cd exposure to establish dose response relationship in the aquatic environment for their survivability. The main haematological alteration resulting from exposure of *A. testudineus* to cadmium include significant decrease in haematocrit and haemoglobin concentration and in red blood cell counts. In case of biochemical parameters, a significant decrease was found in total protein, glucose, albumin, triglycerides content in Cd treated fish while there was an increase in alkaline phosphatase activity. These variations were found to be tissue specific and hence, can be used as meaningful indicators of heavy metal pollution and can interfere with the survivability of *A. testudineus*. The findings of this study may be used as a guideline for biomonitoring programs on fish populations cultured near areas contaminated by heavy metals.

INTRODUCTION

Cadmium (Cd) occurs naturally in the earth's crust and is ranked 7 of ASTDR's "Top 20 list" (ASTDR 2019). During the 20th century, Cd production, consumption and emissions to the environment have increased dramatically due to its industrial use (batteries, electroplating, plastic stabilizers, pigment), and consequently lead to contamination of aquatic habitats (Järup, 2003). Like all metals, Cd can move from one environmental compartment to another and being nondegradable cumulative pollutant, considered to alter aquatic trophic levels for centuries (Sorensen, 1991; Afshan *et al.*, 2014; Authman *et al.*, 2015). Fish can accumulate cadmium from the water and by eating foods contaminated with cadmium (contaminated food chain). In the fish, the possible areas of absorption of dissolved metals are the gills (respiratory tract), the intestine (ingestive intake) and the skin (transcutaneous uptake). There are several reports on the effect of Cd in different freshwater fishes like *Cyprinus carpio, Carassius auratus and Corydoras paleatus* (Mukherjee *et al.*, 1994; Çavaş and Ergene-Gözükara 2005); *Channa punctatus* (Shukla *et al.*, 2002); *Oncorynchus mykiss* (Vétillard and Bailhache 2005); *Oreochromis mossambicus* (van Dyk *et al.*, 2007) to depict the changes in organismal, histological and cellular levels.

In this study, the climbing perch, *Anabas testudineus* (Bloch, 1792) was selected as a test organism as they are abundant in different parts of Asia: Bangladesh, China, India, Malaysia, Myanmar, Pakistan, Philippines, Sri Lanka, and Thailand (Talwar and Jhingran, 1991; Rahman, 2005) and has a high economic value. This potamodromous species mostly dwells in rivers, flooded fields, and stagnant water bodies (slow moving canals) canals, lakes, ponds, swamps, and estuaries (Pethiyagoda, 1991; Vidthayanon, 2002). However, the natural populations are declining rapidly in their natural habitat and aquatic pollution is one of the major reasons for decline in *A. testudineus* population (Kohinoor *et al.*, 2012). *A. testudineus* is an air-breathing fish with high tolerance to hydrological variation. During

reproductive season, brooders climb up to inhabit inundated paddy fields increasing potential risks from Cd exposure. However, Cd is usually found in the aquatic environments at sublethal concentrations (Asagba *et al.*, 2008) and may not provide information on the severity of contamination. Biological monitoring using a series of assays having different endpoints in a key species could be a sensitive approach to predict potential risk of persistent contaminants like Cd, which is helpful in predicting safe levels of such bioaccumulating substance having genotoxic potential. Thus, acute toxicity studies are the very first step in determining the water conditions for survivability of the fish (Parvin *et al.*, 2011). In *Anabas*, the obligatory air-breathing function is performed to high efficiency through specialized labyrinthine organ, a modification of major part of the gills and aided by efficient blood circulation through increased haemopoiesis and vascularization. Hence, in this study our hypothesis has been to identify whether there is any association of Cd (which is prevalent in their habitat) with the physiological stress response leading to their cause of decline. For this we have analyzed different hematological and biochemical parameters in *A. testudineus* along with their behaviour upon acute Cd exposure to establish dose response relationship in the aquatic environment.

MATERIALS AND METHODS

Experimental fish specimens

Adult Specimens of *A. testudineus* (weight of 33.04±0.05 g and length of 11.08±0.34 cm) were procured from local fish farms. The fish were acclimatized under laboratory conditions for 7 days in a flow through recirculatory system with dechlorinated water. The fishes were maintained in normal day-night illumination (14 L: 10D). The physicochemical parameters of the aquarium were maintained as follows: Temperature 25±1ºC, pH 7.2 ±1, DO 6.4±1.5 L^{-1}, ammonia 1± 0.5 ppm, hardness 215±25 mgL^{-1}. The fishes were initially fed with commercial feed purchased from the market followed by earthworms. Fecal matter and other waste materials were siphoned off daily to reduce the ammonia content of the water.

Preparation of Stock Solution

Cadmium chloride of analytical grade was purchased from Sigma Aldrich [Product Code: 202908] and the stock solutions of $CdCl_2$ was prepared by adding correct amount of the salt in Deionized water (1 g L^{-1}). Aliquot volume of desired concentrations was prepared from stock solution and added to the tank through micropipette to give the required concentration of the heavy metal for precision.

Acute toxicity test

Definitive acute toxicity bioassay was conducted by exposing fish to six different concentrations of cadmium, viz., 5, 10, 15, 20, 25 and 30 mgL^{-1} respectively. A simultaneous control group was kept together with the actual experiments, containing the same experimental water without adding cadmium. Acute toxicity tests were conducted in triplicate according to standard toxicity testing protocol (Sprague, 1996) and each experimental group contained twenty fishes. Mortality was recorded on 24, 48, 72 and 96h of exposure. The median lethal concentration (LC_{50}) for *Anabas sp*. at different hours of exposure were estimated by Probit analysis (Finney, 1962) by using the "Descriptive Statistics tool" of Microsoft Excel. To analyze the effects of acute cadmium exposure on *Anabas*, fishes were exposed to $1/5^{th}$ of $^{96hr}LC_{50}$ for 7 days followed by estimation of haemotological and biochemical changes.

Haemogram and Haemopoietic study

The Haematological parameters (Total Erythrocyte count (TEC), Total Leucocyte count (TLC), Haematocrit (Hct %), Haemoglobin %) were estimated by standard methods as described by Dacie and Lewis (1968) and Blaxhall and Daisley (1973). The haemopoietic studies were conducted using the imprint or impression technique (Mahajan and Dheer, 1979, 1980). Imprint of the head kidney tissue was made on multiple glass slides which were air dried and subsequently stained with benzidine (Forteza and Bover, 1964) followed by counterstaining with Giemsa. Morphometry of haemopoietic cells were studied by ocular micrometer in light microscope (Leica DM1000) with camera attachment. Calculation of means, standard deviations (SD) of the means, standard errors (SE) of the means from whole range of data were done using Descriptive Statistics tool of Microsoft Excel 2007.

Analysis of Biochemical parameters and Stress enzyme activity

Whole fishes were euthanized on ice and after that the fins and head were ablated. The body is cut into small pieces and rinsed in ice cold phosphate buffer, PBS (pH 7.4) to remove excess blood. Later the tissue was homogenized and sonicated at 4°C in PBS to prepare a tissue homogenate. The homogenate is centrifuged at 2000-3000 RPM for approximately 20 min at 4°C. The supernatant was stored at -80°C until used for further study. The sample is thawed at 2 to 4°C before using it for analysis. The concentration of all biochemical parameters, Glucose; Total Protein; Urea; Triglyceride; Albumin and the stress enzyme, Alkaline Phosphatase, levels were determined by specific kits from Precision Biomed Pvt. Ltd (Roy *et al.*, 2018). All the assays were done using Robonik Priest Touch Biochemistry Analyser at different filters.

Behavioral response study

The behavioral responses of *Anabas testudineus* was recorded following Rand (1985). The record included convulsions, equilibrium status, fin movement, hyperactivity, opercular movement and surfacing activity.

Statistical analysis:

The significance of differences between control and experimental data was statistically analyzed using Student's t test by SPSS Statistics 17.0. Treatments were taken to be differing significantly where $p<0.05$.

RESULTS

The present study demonstrated the toxic effects of Cadmium on the hematological and biochemical parameters

of the fish. Upon exposure to Cadmium (1/5th of $^{96hr}LC_{50}$ i.e.4.28±0.35 mgL^{-1}) for 7 days, the TEC, TLC, Haematocrit and the Haemoglobin showed marked changes (Fig 1). TEC (7.77±0.14), Hct (0.26±0.8) and Hb (7.43±0.18) decreased significantly ($p<0.05$) in the treated group of fish from the corresponding control values. However, the TLC increased (9.09±0.004) in the treated group ($p<0.05$). The hemopoietic study showed two lineages i.e. erythrocytic lineage, which comprised of eight stages namely small lymphoid haematoblast (SLH), basophilic erythroblast (BE), polychromatophilic erythroblast (PE), acidophilic erythroblast (AE), young reticulocyte (YR), mature reticulocyte (MR), young and mature erythrocyte (YE and ME). Lymphocyte (LYM) and neutrophil (NEU) were identified in the lymphocytic lineage (Fig. 2). The differential cell counts showed significant decrease for SLH (7.26±1.05), BE (2.61±0.02), PE (4.357±0.87),YR (3.53±0.78), MR (7.97 ±1.63),YE (10.552±2.57),ME (25.54 ±1.34). But there is a significant increase in NEU (2.92± 0.31), LLYM (14.75± 0.64), SLYM (9.577±1.93) (Fig 2 & 3). Significant decrease in total protein content in liver (1.0183±0.0014 g/dl), spleen (0.5383±0.006 g/dl), muscle (0.669±0.0064 g/dl) and testis (0.304±0.0046 g/dl) was observed in *A. testudineus* (Fig 4). The urea content showed significant decrease in liver (0.176±0.0009 g/dl), spleen (0.0092±0.0003 g/dl), muscle (0.015±0.0001 g/dl) and testis (0.0143± 0.00015g /dl) ($p<0.05$) (Fig 5). The exposure of Cd induced significant reduction of triglyceride level in liver (0.009±0.0005g/dl), spleen (0.01±0.0005 g/dl), muscle (0.02667±0.002 g/dl), testis (0.0123±0.0008 g/dl) (Fig 6). Cd resulted in a significant decrease in albumin concentrations at metal-exposed fish compared to control groups in case of all the organs (Fig 7). The data of the present study showed that the treated fish liver A significant reduction in the glucose level was detected in liver (0.00489±0.00029 g/dl), spleen (0.00056±0.000004 g/dl), muscle (0.00022±0.00001 g/dl) and testis (0.00017±0.00006 g/dl) in the treated fishes (Fig 8). But the data of the present study showed that the exposure of Cd caused significant elevations in the alkaline phosphatase enzyme activity ($p<0.05$) in all the four organs of treated fishes (Fig 9).

During the study, experimental Cadmium treated fishes exhibited erratic swimming, faster opercular movement, loss of equilibrium and enhanced surfacing behaviour leading to hyperactivity.

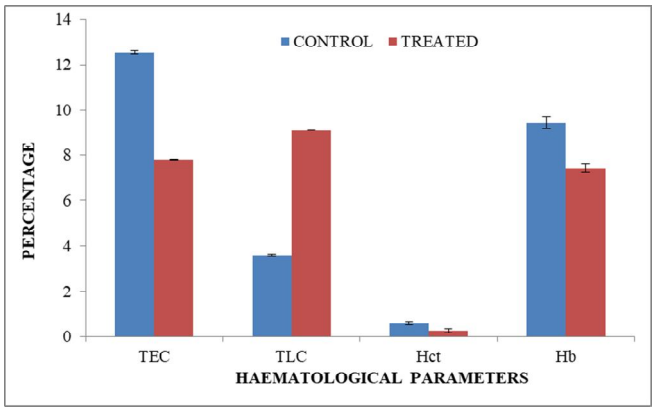
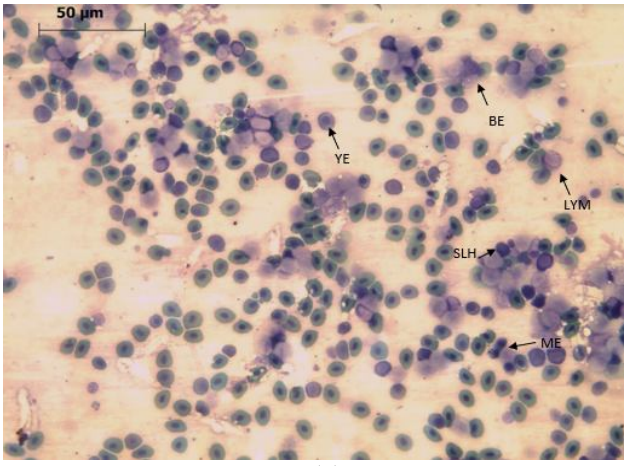
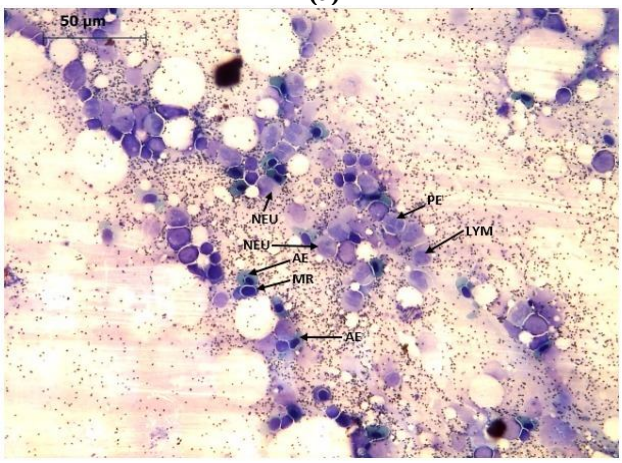


Fig. 1: The haematological changes in *A. testudineus* exposed to cadmium [TEC: Total Erythrocytic count, TLC: Total Leucocyte count, Hct: Hematocrit value, Hb: Hemoglobin]



(a)



(b)

Fig 2: The imprints from head kidney of *A. testudineus* showing (a) Small Lymphoid Haematoblast (SLH), Basophilic Erythroblast (BE), Young Erythroblast (YE), Mature Erythrocyte (ME), Lymphocyte (LYM). (b) Neutrophil (NEU), Acidophilic erythroblast (AE), Polychromatophilic erythroblast (PE), mature reticulocyte (MR), Lymphocyte (LYM)

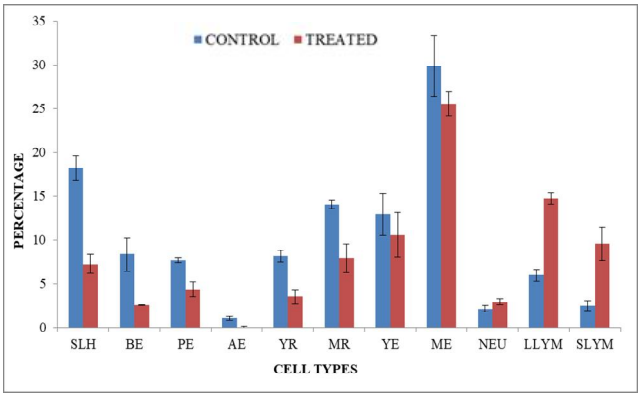


Fig. 3: The effect of cadmium exposure on Hematopoiesis. Abbreviations: Small Lymphoid Haematoblast (SLH), Basophilic Erythroblast (BE), Polychromatophilic Erythroblast (PE), Acidophilic Erythroblast (AE), Young Reticulocyte (YR), Mature Reticulocyte (MR), Young Erythrocyte (YE), Mature Erythrocyte (ME), Lymphocyte (LYM), Neutrophil (NEU)

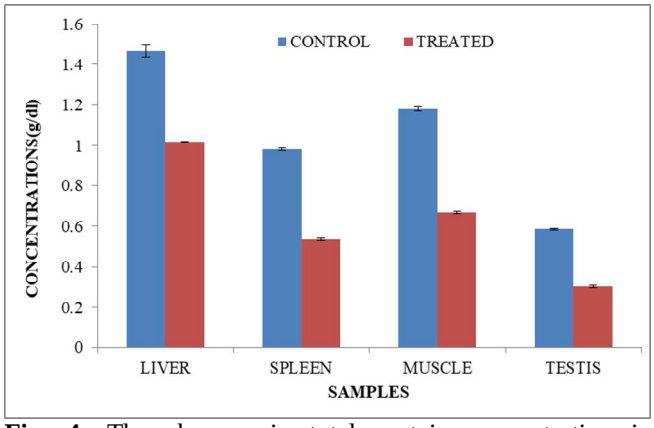


Fig. 4: The changes in total protein concentration in *A.testudineus* under exposure to Cadmium

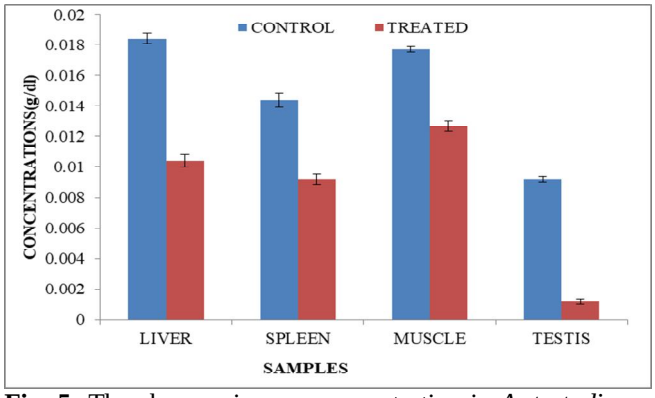


Fig. 5: The changes in urea concentration in *A. testudineus* under exposure to Cadmium

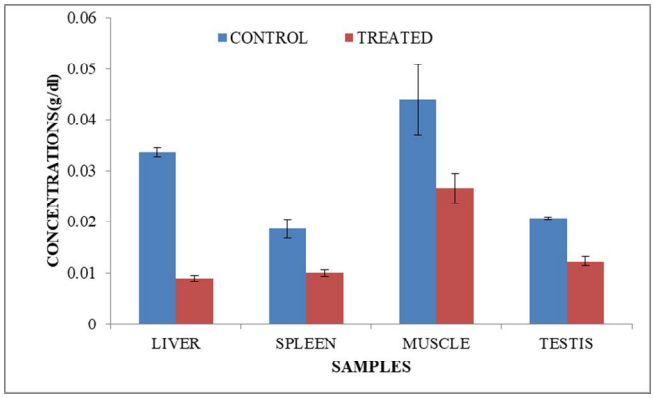


Fig. 6: The changes in triglyceride concentration in *A. testudineus* under exposure to Cadmium

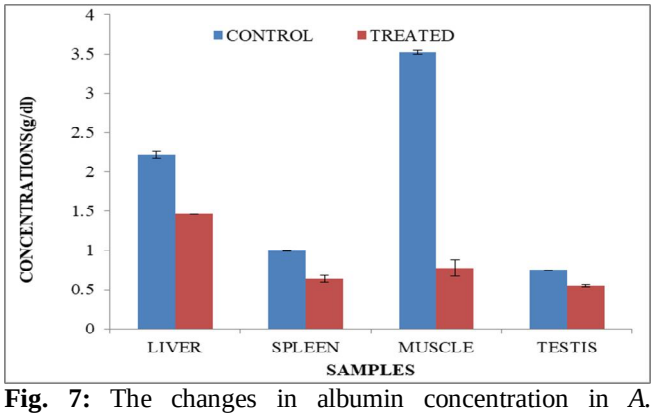


Fig. 7: The changes in albumin concentration in *A. testudineus* under exposure to Cadmium

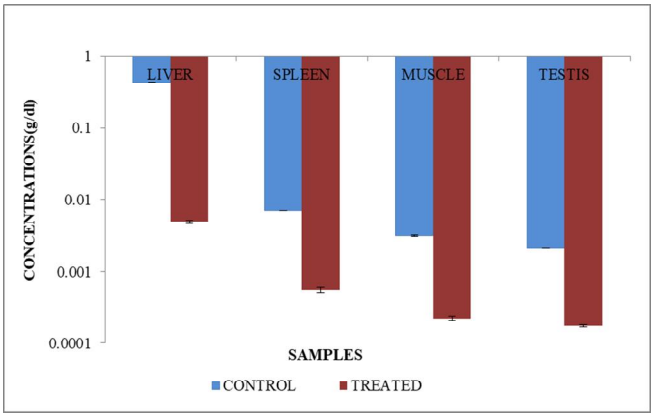


Fig. 8: The changes in glucose concentration in *A. testudineus* under exposure to Cadmium

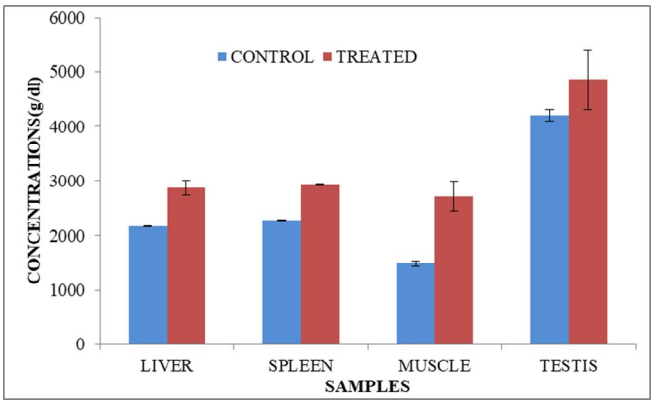


Fig. 9: The changes in alkaline phosphatase concentration in *A. testudineus* under exposure to Cadmium

DISCUSSION

Haematological studies are the reflection of the overall changes that occur in the blood upon exposure to the heavy metal and help to recognize the stresses which occurred due to the treatment (Al-Attar, 2005). In the present study, due to the presence of cadmium in blood, haemolysis occurred which led to the rupturing and destruction of the RBCs. In response to cadmium toxicity towards excessive red cell destruction and reduced hemo synthesis leads to anemia to protect the fish against infections under cadmium stress (Kaoud *et al.*, 2011). Reduction in haemoglobin concentration may probably be due to production of reactive oxygen species under the influence of heavy metal cadmium which results in destruction of the red blood cell membrane and its function (Tariq *et al.*, 1996). Similar observation was found in *Anguilla rostrate* (Gill and Epple, 1993), *Channa punctatus* (Karuppaswamy *et al.*, 2005), *Heteropenustes fossilis* (Bujjamma and Padmavathi 2018). However, in contrary, Witeska and Jezierska (1994) revealed that red blood cell count and haematocrit levels of Cd exposed common carp (*Cyprinus carpio*) increased. The reduction in the total erythrocyte count (TEC) may be due to the cytotoxic effect of heavy metal compounds on the erythropoietic tissue. Such a disturbance in haemopoietic organs leads to alteration of cell cycle and reduction in erythropoeisis (Tariq *et al.*, 1996). Leucocytes or WBC are the cells of immune system which play a key role in both non-specific and specific immune responses in protecting the body against foreign substances and one of the most elementary ways to assess the immune system (Moraes *et al.*, 2007). In

the present study there was a significant increase in TLC as observed in Cd treated fish. Similar finding was observed in different fishes (*Oreochromis mossambicus* (Buthelezi *et al.*, 2000); *Labeo boga* (Raina and Sachar, 2014); *Wallago attu* (Sharma and Langer, 2014); *Cyprinus Carpio Koi* (Ali *et al.*, 2018) on exposure of various heavy metal. Although, Tort *et al.*, (1988) reported that Cd exposure caused leucocyte (WBC) concentration of lesser spotted dogfish (*Scyliorhinus canicula*) to reduce, increase in WBC count can be attributed to a stimulation of the immune system in response to tissue damage caused by heavy metals. An increase in the TLC was also observed due to the uptake of a foreign toxic salt in the blood-stream.

Usually toxicity is a consequence of nonspecific binding of reactive metal cations with biologically important macromolecules causing changes in their functioning. The Biochemical analyses depict the accumulation of the heavy metals in certain vital organs (Liver, Spleen, Muscle and Testis) of the fish and trace the stress or damages caused due to the accumulation. The significant decrease in total protein level in the present study may be due to liver damage, disturbance of cellular fraction, reduction absorption and consequent impairment in protein synthetic machinery (Jipsa and Logaswamy, 2013). Variations in serum triglyceride concentrations might be due to differences in exposure concentration, lipid metabolism, and glycogen storage impairment in different fish species (Heydarnejad et al., 2013). The Cd toxic action upon enzymes is nonspecific and includes blocking of a range of biochemical reactions due to binding of functional –SH groups of proteins or forcing out of trace elements from enzyme active centers. These result in malfunctions of cell metabolism, enhanced lipid peroxidation, inhibition oxidative phosphorylation, damage in Ca^{2+} homeostasis, and structure and permeability of cell membranes Most often highest concentrations of heavy metal are found in fish liver, kidney, gills, and in some cases in the gut (Golovanova, 2008). Hypoalbuminemia was observed in all the organs of cadmium treated fish due to improper liver functioning caused due to the stress (Arya, 2014). Changes of blood glucose are a good indicator of metal stress in fish (Gagnon *et al.*, 2006) and alterations in the glucose level might be related to renal injury, liver damage reflecting the exhaustion of the energy reserves of the organism and an impaired capacity of fish to restore them. This reduction might also be related to renal injury or liver damage and lack of nutrition (Pratap and Bonga 1990; Arya, 2014). It has been shown that Cd exposure caused glucose levels in rainbow trout (*Oncorhynchus mykiss*) to increase (Haux and Larsson, 1984), in common carp (*Cyprinus carpio*) to decrease (Yamawaki *et al.*, 1986). The changes in carbohydrate and lipid metabolisms is associated with the malfunction of liver enzymes activities and similar observations were also revealed in yellow perch *Perca flavescens* (Mitchill) inhabiting waterbodies polluted by different heavy metals Cd, Cu, and Zn (Campbell *et al.*, 2002). The most fundamental device to investigate the physical alterations is the study of changes in the enzyme activity, since this organic cellular catalyst control the formation of biochemical intermediates which are indispensable to all the normal physiological processes. The increased level of alkaline phosphatase may be due to the process of transphosphorylation. Also, uncoupling of oxidative phosphorylation has been the main reason for inhibition of Alkaline phosphatases (Sonawane, 2017).

Swimming performance is considered as behavior parameters to assess the physiological status of aquatic life to measure the presence and effects of contaminant (Ballesteros *et al.*, 2009; Almeida *et al.*, 2010; Cailleaud *et al.*, 2011).The erratic swimming behaviour in *Anabas* after Cd exposure can be corroborated with the finding of Beaumont *et al.*, (1995) who showed that these heavy metal ions are known to have a number of metabolic and physiological effects that may influence swimming performance by interfering with the metabolic status of the muscle or affect central or peripheral nervous activity, transmission at the neuromuscular junction, excitation/contraction coupling or muscle electrophysiology. The reason for faster opercular movement in fish was due to hypoxia caused by exposure of toxins and hyperactivity associated with the inactivation of acetylcholinesterase (Tiwari *et al.*, 2011). Similar observation can be found in the mrigal (*Chirrhinus mrigala*), catla (*Catla catla*) and rohu (*Labeo rohita*) exposed to Cu (Ansari and Kumar, 1984; Venkataramana and Radhakrishnaiah, 2001; Ali *et al.*, 2003; Debnath *et al.*, 2012).

The physiological stress resulting from metal poisoning is thus clearly reflected by blood parameters of the experimental fish *A. testudineus*. The use of immune system parameters to assess alterations in fishes experiencing exposure to heavy metals and its interaction in defense mechanisms stem from the need to develop healthy management tools to support rapidly growing aquaculture industry (Jones, 1983). Since fish lives in close association with their environment and are sensitive to slight fluctuation that may occur within their internal milieu. These altered physiological functions in fish hematology and biochemical parameters thus can be associated with toxin contaminated water quality **(**Omoregie *et al.*, 1990; Srivastava and Punia, 2011).

CONCLUSION

The present investigation emphasized the usefulness of fish blood and organs as an experimental model for studying the effects of aquatic environmental toxicants on fish. As the circulatory system of fish is in close association with the external environment and with every tissue. The findings of this study could be used as a guideline for biomonitoring programmes on fish populations cultured near areas contaminated by heavy metals. It was observed that there is a significant decrease in the Erythrocytes and increase in the Leucocytes. The lower value of the hematological parameters may be due to disturbance of this osmoregulatory mechanism accompanied with destruction of gill membrane and failure of gas exchange and significant higher values of leucocyte is to defend the inflammatory changes (Al-Attar, 2005). The present work revealed that the variations in bio-chemical parameters serve as indices in monitoring the pathological status of the heavy metal treated fish. These variations were found to be tissue specific and hence, can be used as meaningful indicators of heavy metal pollution and can interfere with the survivability of *A. testudineus*. The alteration in the biochemical profile reflected the capacity of the fishes to cope with the stressed condition. As majority of heavy metals are released by the industrial and anthropogenic activities, their residues accumulate in the tissues of fish,

and transfer via food chain and increase the risk to the health of the people while consuming these fishes. Hence, there is an urgent need to protect people from this undue exposure by minimizing and responsible use of the heavy metals.

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