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Alteration of Fish Physiology as Indicator of Anthropogenic Stress

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Abstract: **Anthropogenic stresses alter different biochemical and physiological parameters in an organism. Measurement of these changes can be used to assess the health of the fish under study. In this study fishes (*Labeo rohita*) were collected from three different wetlands (Bhomra beel, Mathura beel and Kalyani lake), situated in the same geographical region but possessing different levels of anthropogenic threats. Fish health was monitored by examining changes in blood hematological parameters (Red blood cell count, hemoglobin, mean cellular hemoglobin content, mean cellular hemoglobin concentration, WBC count) as well as hepatic superoxide dismutase (SOD) activity & liver histology. SOD showed significant up regulation in response to oxidative stress. In this comparative study SOD activity showed more changes in mostly polluted wetland i.e., Kalyani Lake. In this study, liver histology showed few notable changes. Level of anaemia and leucocytosis was maximum in Kalyani lake and minimum in Bhomra beel. Comparison of liver histology among the three beels revealed more distinct apoptotic & necrotic changes along with periductular fibrosis and melanomacrophage centers in carps' liver tissue samples collected from Kalyani Lake. It further reinforces the hypothesis of presence of more stressors in that particular polluted wetland i.e., Kalyani Lake.**

Keywords: **Anthropogenic, Haematological, Polluted, Leucocytosis, Melanomacrophage, Anaemia, Superoxide dismutase.**

I. INTRODUCTION

Biomonitoring is the science of assessing the condition of an environment or ecosystem through observations conducted on the biota. Fish, and their responses, have been extensively used as indicators of habitat quality in assessment tools developed for scientific and management purposes (10 & 3). Haematological techniques are the most common methods to determine the sub-lethal effects of the pollutants (1). It provides a quick screening method, as changes in blood parameters are often quick response to environmental or physiological stresses (14).Other biochemical parameters including antioxidant defense system i.e., tissue Superoxide dismutase (SOD)also represent fine tools for evaluating the effects of contaminants and for environmental monitoring (6) . As an indicator of pollution, thus blood parameters such as RBC (Red Blood Cells), Hb (Haemoglobin), PCV (Packed Cell Volume), TLC (Total Leucocyte Count), MCH (Mean Corpuscular Haemoglobin), MCHC (Mean Corpuscular Haemoglobin Concentration) are studied to diagnose and describe the general health condition of fish. Studies showed a significant change in the hematology of the common fresh water fish on exposure to pollutants. The total leucocyte count (TLC) variation was reported in case of organophosphorous pesticides (9). Many pollutants can induce the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2•) and hydroxyl radical (OH•). Due to their high reactivity, these species may damage lipids, proteins, carbohydrates and nucleic acids (11).

In the present study, we selected three wetlands (The Bhomra beel, the Mathura beel and the Kalyani Lake) in the same geographical area (Ganga delta plain). All the three beels are rich in fish production. Bhomra beel and Kalyani Lake are

situated in the district Nadia and Mathura beel stretching both in Nadia and north 24- parganas districts (West Bengal, India). Bhomra beel is currently threatened by different anthropogenic activities, gradually inclining towards eutrophication. The water quality of the Mathura beel is deteriorating at an alarming rate. It receives both municipal and domestic sewage from Kanchrapara Township.

Kalyani is a fast developing industrial town with increasing commercial activities. The connections of Kalyani Lake with the river Ganga do not exist anymore, causing rapid trophic status change due to fast urbanization resulting in heavy weed infestation. In our study we focused our attention to hematological parameters, activity of one antioxidant enzyme in hepatic tissues, i.e., SOD, and Liver Histology of *Labeo rohita* collected from different study areas of three beels. The aim of this research was to investigate the use of fish physiological and biochemical responses as indicators of habitat quality in the environment for a period of 3 yrs (2009-2011).

II. MATERIALS AND METHODS

Male *Labeo rohita* were chosen for this study to prevent bias which might be introduced due to sex hormones. The carps included in this study were weighed between 350-450g.

Limnological Analysis:

Temperature, pH, Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Free Carbon dioxide (Free CO2), Nitrate, Phosphate, Hardness were measured following standard protocols in the all three water bodies included in this study.

Sample Collection and Transport:

Venous blood was collected from the caudal vein by using heparinized hypodermic syringe & transferred to EDTA tubes, BD, India and dissection was started from the anal fin with a scalpel. The liver was dissected out. First the tissue was placed in cold normal saline and subsequently one part was transferred in cold Tris-HCl buffer (pH 7.4) for SOD estimation & another part in 10% formalin for histopathological examinations.

All the samples were transported to the laboratory via insulated thermocol box within 4 hours of collection.

Histological Study:

Standard histological protocol was followed. Each type of change was observed & recorded (Figs. 2,3,4 & 5)

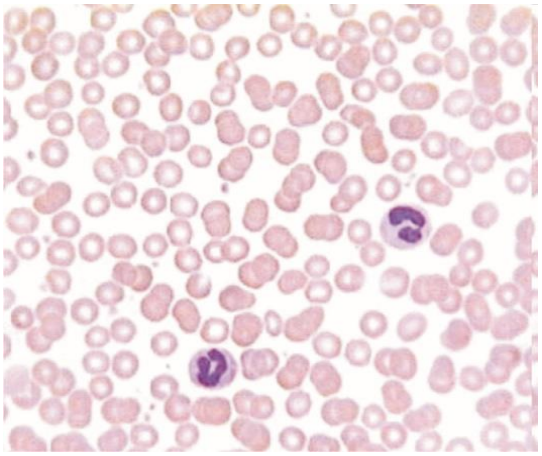

Fig.2: Shows Normocytic Normochromic picture with adequate RBC count (sample from Bhomra Beel).

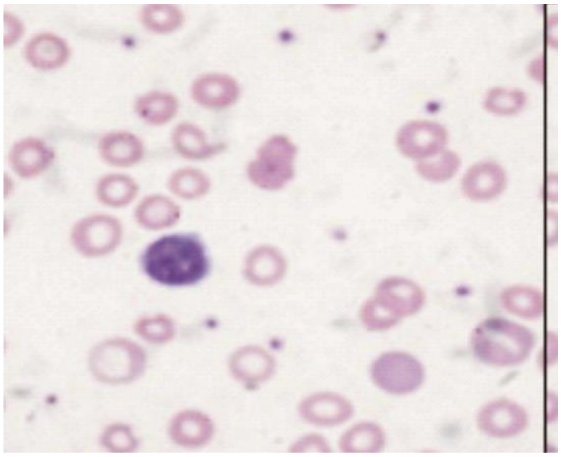

Fig.3: Shows microcytic hypochromic picture of RBC; smear drawn from a sample collected from Kalyani Lake.

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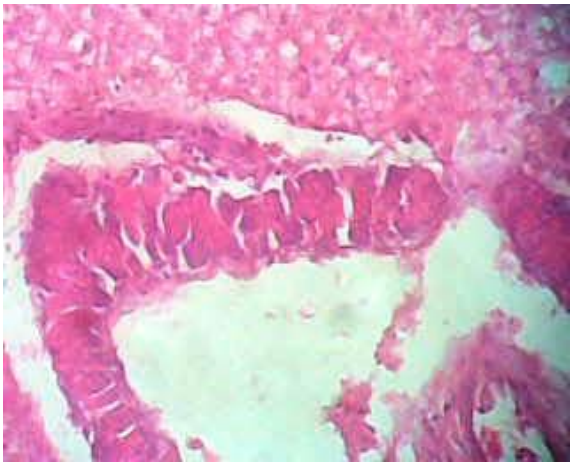

Fig.4: Shows normal structures in the hepatic tissue of the dissected carp *(L.rohita)* collected from Bhomra Beel. Figure also shows more nonresident perisinusoidal macrophages and hepatic stellate cells compared to mammals.

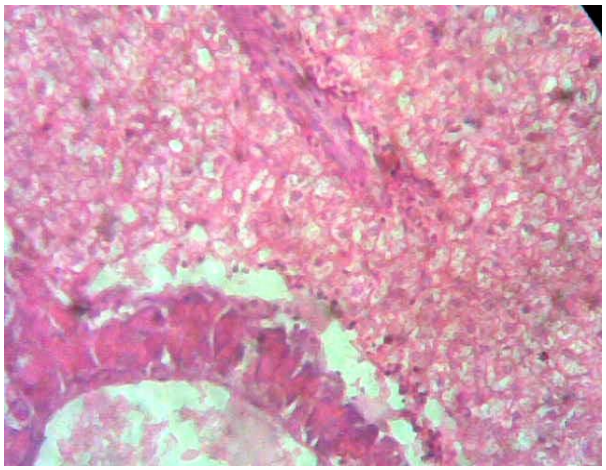

Fig.5: Shows more apoptotic & necrotic sites within the hepatic tissue from the carp collected from Kalyani Lake.

Haematological Assays:

The peripheral blood smears were dried & placed properly for Leishman's stain, BIOLAB, India. The stained smears were examined by both 40X &100X lenses of Olympus CHOi, Olympus, USA & LABOMED LX 300 light Microscopes. The other portion of anticoagulant mixed blood was aspirated through semiautomated cell counter [Medonic-SN 14592, Merck, USA].Haemoglobin was estimated by cyanomethemoglobin method by the instrument (17) RBC Count, TLC, differential WBC count & other red cell indices i.e., MCH, MCHC were done by aperture impedance technique and calculated by the instrument. The differential WBC counts were verified by examining peripheral blood smear. RBC count & TLC counts were further checked by Neubauer's Haemocytometer with Toissions solution as diluting fluid for TEC and Turk's solution for TLC.

Biochemical Assays:

The fish liver tissue collected in cold Tris-HCl buffer (pH 7.4) was first homogenized by simple motorized Homogenizer and then centrifuged in REMI Cold Centrifuge, REMI, India, for estimation.

SOD Estimation:

SOD estimation was done as described by Kakkar's method (15). It was based on Chromogen production using phenozinemethosulphate (PMS),Sigma-Aldrich, UK, nitro blue tetrazolium salt (NBT),Sigma-Aldrich, UK and NADH,Sigma-Aldrich, UK in presence of SOD enzyme in tissue homogenate. SOD activity was expressed against per gm protein present in liver homogenate. The results of SOD activity have been plotted in Fig.No.1. (Fig.1.).

Estimation of Hepatic Tissue Protein:

Tissue protein estimation was done as described by Folin-Phenol, BIOLAB, India, method (13). Concentration of protein in liver tissue was expressed in mg. ml^{-1} after correcting the dilution factor.

Statistical Analysis:

Pearson's correlation of coefficients was calculated using SPSS (Version 16) between limnological and haematological parameters and SOD. The 'r' values obtained after statistical analysis reveal the correlation between the parameters. Values were arranged to test the correlation at 5% and 1% levels of significance.

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III. RESULTS

Data obtained for Hematological parameters & antioxidant enzyme (SOD) from three different wetlands are represented in Table I.

The mean haemoglobin of the carp samples showed decreasing trend during the study period (from 6.46 ± 0.14g.dL^{-1} in 2009-10 to 6.33 ± 0.14g.dL^{-1} in 2010-11). The mean TEC of the carps' samples collected on 2009-10 period was decreased to 3.90 ± 0.11 millions.cumm^{-1} (mean ± SD) in 2010-11 period from 4.03 ± 0.09 millions.cumm-^{1} in 2009-2010 (Table1) indicating increasing contamination probably adversely affected erythropoeisis in particular water body.PCV was decreased to 36.25 ± 0.96% (mean ± SD) in 2010-11 period indicating increase in the level of pollution (Table I).The MCHC and the MCH values also showed similar trend as these are derived parameters. But the mean TLC and the SOD activity showed reverse trend during the study period (Table I).

Table I: Hematological parameters and Superoxide dismutase (SOD) of *Labeo rohita* collected from three selected wetlands during study period (2009-2011).

Hematological parameters & Antioxidant enzyme activity	Bhomra Beel		Mathura Beel		Kalyani Lake	
	2009-10	2010-11	2009-10	2010-11	2009-10	2010-11
HB(g.dL$^{-1)}$	6.46 ±0.14*	6.33 ±0.14	6.28 ±0.13	6.17 ±0.10	5.95 ±0.14	5.86 ±0.17
RBC(millions. cumm$^{-1)}$	4.03 ±0.09	3.90 ±0.11	3.85 ±0.12	3.77 ±0.12	3.71 ±0.15	3.62 ±0.13
PCV(%)	37.5 ±1.09	36.25 ±0.96	35.42 ±0.67	34.67 ±0.78	34.25 ±0.96	34 ±0.85
MCHC(g.L^{-1})	172.53 ±4.73	174.76 ±3.30	177.42 ±2.57	178.19 ±4.36	173.79 ±4.19	172.32 ±3.60
MCH(pg)	16.04 ±0.35	16.21 ±0.34	16.33 ±0.36	16.37 ±0.37	16.03 ±0.59	16.20 ±0.32
TLC(x1000. cumm^{-1})	9.15 ±0.44	9.25 ±0.18	9.42 ±0.22	9.53 ±0.29	9.60 ±0.25	9.90 ±0.19
SOD(U.mg protein$^{-1)}$	211.83 ±15.5	241.25 ±26.81	333.33 ±33.66	395.42 ±37.87	486.25 ±35.36	557.08 ±30.85

*(Mean±SD)

The rapid eutrophication & pollution might have an impact on TLC. The activity of SOD was upregulated in hepatic tissue (from 211.83±15.58U.mgprotein^{-1} in 2009-10 to 241.25±26.81U.mg protein-^{1} in 2010-11) to combat increased oxidative stress (Fig.1).

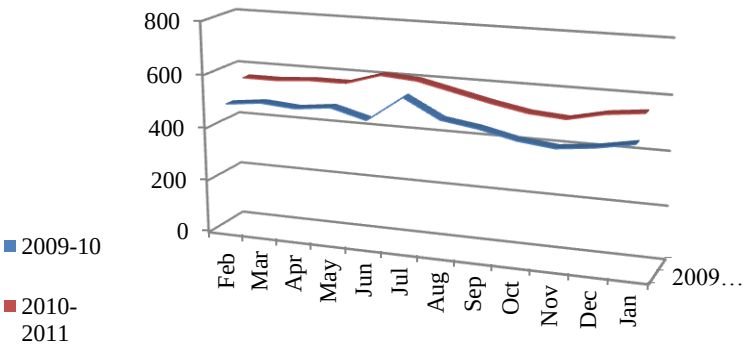

Fig.1: Shows comparative monthly fluctuation of SOD activity (U.mg protein^{-1}) in Kalyani Lake during study period (2009-10 & 2010-11) (X axis: month & Y axis: SOD activity).

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The mean haemoglobin of the carps' samples fished on 2009-10 period was 6.28±0.13 $g.dL^{-1}$ (mean±SD). It was decreased to 6.17±0.10$g.dL^{-1}$ in 2010-11 periods. The mean TEC of the carps' samples collected on 2009-10 period was 3.85 ± 0.12millions.$cumm^{-1}$ (mean ± SD). It was decreased to 3.77 ± 0.12 millions.$cumm^{-1}$ (mean ± SD) in 2010-11 period .The mean PCV was decreased to 34.67 ± 0.78% (mean ± SD) in 2010-11 from 35.42 ± 0.67% in 2009-10 periods.

The mean TLC was also increased from 9.42 ± 0.22 × 10^3.$cumm^{-1}$ (mean ± SD) in 2009-2010 to 9.53 ± 0.29 × 10^3.$cumm^{-1}$ (mean ± SD) in 2010-11(Table I). Similarly the mean SOD activity of the carps' samples collected showed increasing trend during the study period (Table I).

The mean haemoglobin of the carps' samples was decreased (5.95 ± 0.14 $g.dL^{-1}$ to 5.86 ± 0.17$g.dL^{-1}$) during the study period. Similar findings were also observed in case of TEC and the mean PCV or Haematocrit value (Table I). While the mean TLC and the mean SOD activity of the carps' sample showed increasing values in the second year of our study (Table I).

Statistical Analysis:

The Pearson's correlations of coefficients obtained after statistical analysis are tabulated in the Tables (Table: II-IV). In case of Bhomra beel, haemoglobin showed negative correlation with most of the limnological parameters. BOD was found to be the major contributing factor(r = - 0.770**). In Mathura beel, most physicochemical parameters were associated in negative correlation with Hb, BOD displaying the highest r value (-0.728**). PCV had significant correlation with limnological parameters. TLC was in positive correlation with most of the parameters. pH had no correlations at all (Table III).While in Kalyani lake, Hb% displayed negative correlation with most of the limnological parameters (maximum r = 0.629** with BOD). PCV showed significant positive correlation with most of the parameters studied. Strong negative correlation was observed .

Table II: Correlation between physiological & limnological parameters of Bhomra Beel

Limnological parameters	Hb	PCV	RBC	TLC	MCH	MCHC	SOD
BOD#	-0.761**	-0.770**	-0.661**	0.607**	0.053	0.277	0.822**
FREE CO_2#	-0.717**	-0.703**	-0.444*	0.779**	-0.192	0.233	0.698**
WT#	-0.333	-0.472*	-0.164	0.450*	-0.143	0.304	0.468*
NITRATE	0.080	-0.337	0.035	0.201	0.041	0.536**	0.400
PO_4#	-0.689**	-0.534**	-0.461*	0.838**	-0.130	0.027	0.822**
COD#	-0.739**	-0.621**	0.596**-	0.628**	-0.012	-0.092	0.678**
HARDNESS#	-0.841**	-0.597**	-0.632**	0.674**	-0.075	-0.036	0.556**
pH	-0.729**	-0.405*	-0.485*	0.641**	-0.146	-0.176	0.530**

Vide Table of abbreviations. ** Correlation is significant at 0.01 level (2-tailed). * Correlation is significant at 0.05 level (2-tailed)

Table III: Correlation between physiological & limnological parameters of Mathura Beel.

Limnological parameters	Hb	PCV	RBC	TLC	MCH	MCHC	SOD
BOD#	-0.728**	-0.611**	-0.733**	0.712**	0.384	-0.006	0.752**
FCO_2#	-0.581**	-0.589**	-0.641**	0.657**	0.388	0.122	0.706**
WT#	-0.269	-0.208	-0.560**	0.682**	0.548**	-0.021	0.388
NO_3#	-0.091	-0.194	-0.334	0.545**	0.396	0.136	0.681**
PO4#	-0.362	-0.298	0.567**-	0.655**	0.477*	-0.004	0.293
COD#	-0.708**	-0.529**	-0.720**	0.530**	0.387	-0.079	0.644**
HARDNESS#	-0.724**	-0.598**	-0.583**	0.565**	0.178	-0.019	0.722**
pH	-0.461*	-0.122	-0.315	0.129	0.028	-0.318	-0.128

Vide Table of abbreviations.** Correlation is significant at 0.01 level (2-tailed). * Correlation is significant at 0.05 level (2-tailed)

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Table IV: Correlation between physiological & limnological parameters of Kalyani Lake.

Limnological parameters	Hb	PCV	RBC	TLC	MCH	MCHC	SOD
BOD[#]	-0.629**	-0.747**	−0.839**	0.541**	0.490*	0.032	0.574**
FREE CO2[#]	-0.439**	-0.583**	−0.630**	0.742**	0.357	0.036	0.750**
WT[#]	-0.648**	-0.670**	−0.564**	0.267	0.424*	0.338	0.237
NITRITE[#]	-0.322	-0.452*	−0.551**	0.746**	0.476*	0.167	0.688**
PO4[#]	-0.614**	-0.641**	−0.706**	0.745**	0.400	0.020	0.646**
COD[#]	-0.250	-0.578**	−0.644**	0.691**	0.425*	0.084	0.775**
HARDNESS[#]	-0.602**	-0.601**	−0.814**	0.561**	0.500*	-0.087	0.570**
pH	-0.252	-0.444*	−0.325	-0.155	0.237	0.242	0.026

[#] Vide Table of abbreviations. ** Correlation is significant at 0.01 level (2-tailed). * Correlation is significant at 0.05 level (2-tailed)

With RBC, BOD (-0.839**), hardness (-0.814**) No significant correlation was observed with MCHC and limnological parameters studied in this investigation of this lake (Table IV).

IV. DISCUSSION

The significant decrease in the hemoglobin concentration and RBC count in the present study was an indicator of either red cell swelling and or decrease in hemoglobin synthesis. The authors in (7) reported that prolonged reduction in hemoglobin content was deleterious to oxygen transport and any blood dyscrasia and degeneration of the erythrocytes could be ascribed as pathological conditions in fishes exposed to toxicants or infectious diseases.

The removal of xenobiotics, and even some endogenous substances, from the cell is catalyzed by a number of different enzymes (Phase I and II). The activity of phase I enzymes can lead to an increase in ROS production or the generation of reactive, redox cycling intermediates. Antioxidant enzymes facilitate the removal of these reactive chemical intermediates and resulting ROS molecules. The action of CYP1A can result in the production of $O_2^{\bullet}$ which in turn can be metabolized by superoxide dismutase (SOD) to H_2O_2. This hydrogen peroxide molecule can then be reduced to H_2O and O_2 by catalase (CAT). This highly potent hydroxyl radical can attack both protein and lipid molecules to form oxidative damage products.

In the cell $O_2^{\cdot}$ is produced at any location where an electron transport chain is present.H_2O_2 can induce intra-molecular disulfide linkages or protein dimerization via intermolecular disulfide linkages. Protein kinases may dimerize or bind/unbind regulatory proteins, thereby altering activity (18).

Increase in the activity of SOD is usually observed in the face of environmental pollutants (2). As found in the present study, SOD activity is upregulated in hepatic tissue in oxidative stress. It is further evident when we compare the three beels under current study. The SOD is over expressed in the hepatic tissue of the carps captured from Kalyani Lake. The mean SOD value of hepatic tissues was lowest in the fishes captured from Bhomra beel and highest in Kalyani lake. Again the mean values of SOD are increased in all the three water bodies from the study year 2009 - 10 to 2010 - 11, because of increasing environmental stress due to rapid urbanization.

Reduction of total erythrocytes count indicated that *Labeo rohita* exposed to sublethal concentration of pollutants in water became anemic (5). The decrease in TEC may be due to the adverse effect of pollutants and oxidative stress on the haemopoietic stem cells (16). The decrease in PCV values can be well explained in the light of decreased Hb% and TEC In the present study the mean Hb%, TEC & PCV values were highest in Bhomra Beel, lowest in Kalyani Lake. The Mathura Beel is sandwiched in between. The physicochemical parameters of Bhomra Beel were not too stressful to the aquatic fauna. But if when the two study years (2009 - 10 & 2010 - 11) were compared, it was obvious that the water quality of all the three wetlands have deteriorated rapidly. There was suggestive of appreciable decline in the haematopoiesis leading to various types of anemia and deformed RBC morphology e.g., microcytic, poikilocythemic, anisocytic anemia. The circulating erythrocytes were immature, varied in size and shape. The nuclei also were varied in size and shape i.e., roundish, swollen, irregular and pyknotic.

The MCV gives an indication of the status or size of the erythrocytes and reflects an abnormal cell division during erythropoiesis. The decrease in MCV that was observed in the samples collected from more polluted waterbody correlates well with oxidative stress and with low haemoglobin content. This microcytosis may be linked with hypoxia. This microcytic anemia was the cause of low haematocrit or PCV values. Similar pattern has been observed in *Labeo umbratus* after exposure to various pollutants (4).

The significant decrease in MCHC in the present study was an indicator of either red cell swelling and or due to a decrease in haemoglobin synthesis. The author (12) reported that prolonged reduction in haemoglobin content was deleterious to oxygen transport and any blood dyscrasia and degeneration of the erythrocytes could be ascribed as pathological conditions in fishes exposed to toxicants or infectious diseases.

There was significant increase in the TLC in Kalyani Lake in comparison to Bhomra Beel & Mathura Beel. The WBC count is lowest in the samples collected from Bhomra Beel. Again there is further increase in TLC count from the year 2009 - 10 to 2010 - 11 as the level of pollution has increased in all the three water bodies of this present study. The total number of WBC is an important indicator of health or disease in fishes.

Histopathology of liver of carps showed granules in the cytoplasm. Bile stagnation was identified as brownish-yellow granules in the cytoplasm. Cytoplasmic and nuclear degeneration was also very common; melanomacrophages were identified as rounded aggregates of cells containing dark-yellowish granules of various sizes, normally close to the vessels (19) along with periductular fibrosis (8). Lesions such as increase in perivascular tissue, hydropic change and vacuolation in the hepatocytes were observed. The majority of the alterations found in the liver of the fishes were collected from Kalyani Lake. More apoptotic & necrotic sites within the hepatic tissue were also observed (20). The liver tissue collected from the fishes of the Kalyani Lake was moderately damaged, and recuperation was still possible, if the water quality improved.

V. CONCLUSION

The physiological and biochemical parameters of *Labeo rohita* have been investigated in three water bodies, i.e. Bhomra beel, Mathura beel & Kalyani lake with different anthropogenic stressful situations. The severity of the changes have been noticed especially within the fishes of the Kalyani Lake due to the stressful situation i.e., due to more polluted conditions.

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