Toxicological Studies of Herbicide Pyrazosulfuron-Ethyl on Fresh Water Fish: A Sub-Acute Study

Research Synopsis for Ph.D.
Submitted to
Department of Zoology
The Maharaja Sayajirao University of Baroda, Vadodara
By

Ankur A. Upadhyay

Guide
Dr. P. H. Parikh
Professor, Department of Zoology,
The Maharaja Sayajirao University of Baroda,
Vadodara - 390002
Gujarat, India

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INTRODUCTION:

Pests and diseases, the prime factors causing low agricultural productivity, are mostly controlled by chemical means. Indiscriminate use of pesticidesto boost crop production with the sole aim of getting more yields is common practices which result in serious environmental hazards. Fresh Water bodies like ponds, lakes, river and low lying water areas are continuously getting polluted. These ecosystems are the ultimate descend of natural and anthropogenic inputs of contaminants into the environment. The agrochemicals used in agriculture are posing a great threat to aquatic fauna especially to fishes, which constitute one of the major sources of protein rich food for mankind.

The world market on pesticides is estimated to grow @ 4.5% each year with the largest growth occurring in herbicides. The average growth rate in Asia Pacific region is approximately 5–7%, but in Indonesia and Pakistan, the market is expanding @ 20-30% per annum. Along with the increase in the amount of pesticide consumption, there is a change in the potency of some new chemicals observed in recent past (Mishra, 2011).Herbicides (plant poisons) are commonly used to manage land and water plants. Herbicides are relatively easy to apply and are the only practical method of control in some situations. However, the treatment of weed-infested waters with herbicides must be used with caution particularly with aquatic life as they can be toxic to fish and other aquatic life. Herbicides are the most commonly used pesticides, and are the most often detected in surface waters. Numerous commercial formulations containing different herbicides (glyphosate, paraquat, sulfonolureaetc) have become popular around the world due to their effective action and low toxicity to mammals. However; they have proved to be harmful to the environment. The herbicides 2, 4- D Sodium salt is selective in nature, glyphosate and paraquat are of non-selective and are used as post emergence herbicides. It is contributed though several herbicides used in India approximately 50 % of agricultural areas including atrazine, diuron, isoproturon. The 2, 4-D Sodium salt is dichlorophenoxy-acetic acid, Phonoxy acids group, glyphosate is N- (Phosphonomethyl) Glycine, organophosphorus group and paraquat is 1, 1-dimethyl-4, 4- bipyridilium dichloride, bipyridylium group (Deivasigamani, 2013) . These herbicides are used in aquatic ecosystem and are easily degraded in water system compared to other herbicides. The acute toxicity of glyphosate is considered to be low, according to data from the WHO (Oleh*et al*., 2009). The toxicity and risk for humans, other mammals and

birds is analyzed by Williams and colleagues (2000). Glyphosate also known to have negative impact on energy metabolism, free radical processes, acetylcholine esterase activity (Langiano*et al.,* 2008)and immune responses of histological changes in hepatocytes of *Oreochromisniloticus*(Szarek*et al*.,(2000) and *Cyprinuscarpio* (Jiraungkoorskul*et al*., 2003).

Sulfonylurea herbicides are an important class of herbicides used worldwide for controlling weeds in all major agronomic crops. Among sulfonylurea products, pyrazosulfuron-ethyl (PE) herbicide is widely used for selective post-emergence control of annual and perennial grasses and broadleaved weeds in cereals. PE is widely used in rice crops in India and is currently recommended for use on some relevant crops in over 30 countries (Singh *et al*., 2012; Giovanni *et al*., 2011). Due its widespread use, it has become a potential water pollutant and presents environmental risk, especially for aquatic organisms, owing to its fairly high water solubility which result in its high mobility. It has been detected in surface and groundwater (Battaglin*et al.*, 2000). Phytotoxicity of chlorsulfuron, sulfometuron-methyl and metsulfuron-methyl has been reported for higher plants (Sabater and Carrasco, 1997). Toxicity of triasulfuron on aquatic organisms has been reported earlier (Baghfalaki*et al*., 2012***).***

Herbicides at high concentration are known to reduce the survival, growth and reproduction of fish, and produce many visible effects on fish (Rahman*et al*., 2002).Normally these pesticides reach the aquatic environment through surface runoff, sediment transport from treated soil and direct application as a spray to water bodies to control the inhabiting pests. Herbicides can accumulate in bodies of water to levels that kill off zooplankton, the main source of food for young fish.Contamination of surface waters by herbicides derived from agricultural premises has become a serious problem Worldwide (Oruc and Uner, 1999; Grzegorz*et al*., 2012). Carps exposed to 5mg/L and 10mg/L of glyphosate for two weeks had gill and liver damage respectively (Neskovic*et al*., 1996). Herbicides disrupt ecological balance affecting non-target organisms including fish (Bretand*et al*., 2000; Oruc and Uner, 1999).Toxicity testing of chemicals on animals has been used for a long time to detect the potential hazards posed by chemicals to man. Bioassay technique has been the cornerstone of programmes on environmental health and chemical safety (Ward and Parrish, 1982).

Aquatic bioassays are necessary in water pollution control to determine whether a potential toxicant is dangerous to aquatic life and if so, to find the relationship between the toxicant concentration and its effect on aquatic animals. Thus, the study is taken up to evaluate the

toxicity of PE on fresh water chichlidO.mossambicus and to assess its potential in altering the physiological orchestration of the model organism.

Materials and Methods:

Animal Model: Fish *Oreochromis mossambicus*

The specimens of freshwater fish, *O. mossambicus* of similar size in length around 12 ± 2 cmand weight 25 ± 1.9 g will be brought from a localpond of Baroda district. The fishes will be acclimatizedin laboratory conditions in well aerated dechlorinatedtap water.

Table 1: General Experimental design

Chemical used	Dosage	Time of exposure	Species
Pyrazosulfuron Ethyl (Herbicide)	Determination of LC_{50} Value ($1/5^{th}$ LC_{50}, $1/10^{th}$ LC_{50} and $1/20^{th}$ LC_{50})	7 and14 days	*Oreochromis mossambicus*

<u>Objective 1:</u>To check the acute toxicity and sub-acute toxicity of Pyrazosulfuron Ethyl to *O.mossambicus.*

Healthy fishes *O. mossambicus* were selected for the test (N=10) to determine the LC_{50} value of fish. Based on the pilot experiments, the experiment was conducted to determine the toxicity in different concentrations. The Preliminary range finding concentrations that were used according to OECD and EPA guidelines are 50 mg/l, 100 mg/l, 200 mg/l, 300 mg/l, 400 mg/l, 500 mg/l, 600 mg/l, 700 mg/l, 800 mg/l, 900 mg/l and 1000 mg/l with three replicates each. The stock solutions were prepared and the required quantity of PE was drawn from the stock solution to find out the LC_{50} values for 96h.

Thus for acute study the fishes were divided into various groups: Group 1 served as control, while Group 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 were treated with PE with concentration 50 mg/l, 100 mg/l, 200 mg/l, 300 mg/l, 400 mg/l, 500 mg/l, 600 mg/l, 700 mg/l, 800 mg/l, 900 mg/l and1000 mg/l, respectively. The control and the exposed fish were aerated frequently to prevent hypoxic condition of the medium. Feeding to fishes was stopped during the experiment. Mortality of the fish was recorded from time to time till 96 hours.

Probit analysis was used to calculate the medium lethal concentration and time with their upper and lower confident limit (Fig 1and 2).

Probit analysis revealed that LC_{50}value increased with increase concentration depicting a direct correlation between mortality and concentration.96hrs LC_{50} value along with its 95% lower and upper confidential limit (LCL and UCL) for PE are represented in Table 2.Through Probit analysis LC_{50} value for PE was found to be**500mg/L.**After obtaining LC_{50} value the sub-acute dose of PE was decided i.e. $1/5^{th}$ of 96 h LC_{50} of PE (100 mg/L; Treatment-I-HD), $1/10^{th}$ of 96 h LC_{50} of PE (50 mg/L; Treatment-II-MD) and $1/20^{th}$ of 96 h LC_{50} of PE (25 mg/L; Treatment-III-LD).

Fig 1: Plot of adjusted probits and predicted regression line of PE to *O.mossambicus*

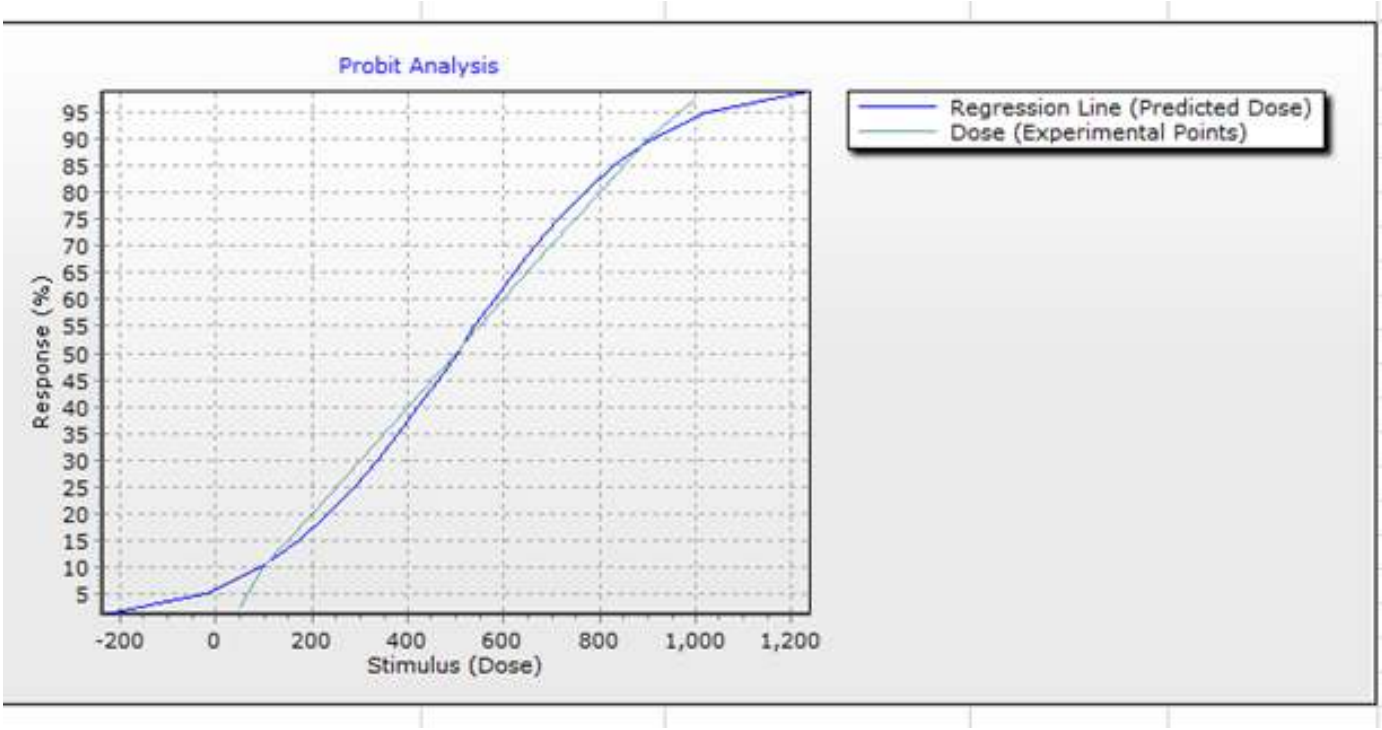


Fig 2:Plot of adjusted probits and predicted regression line of PE to *O.mossambicus* in log10base

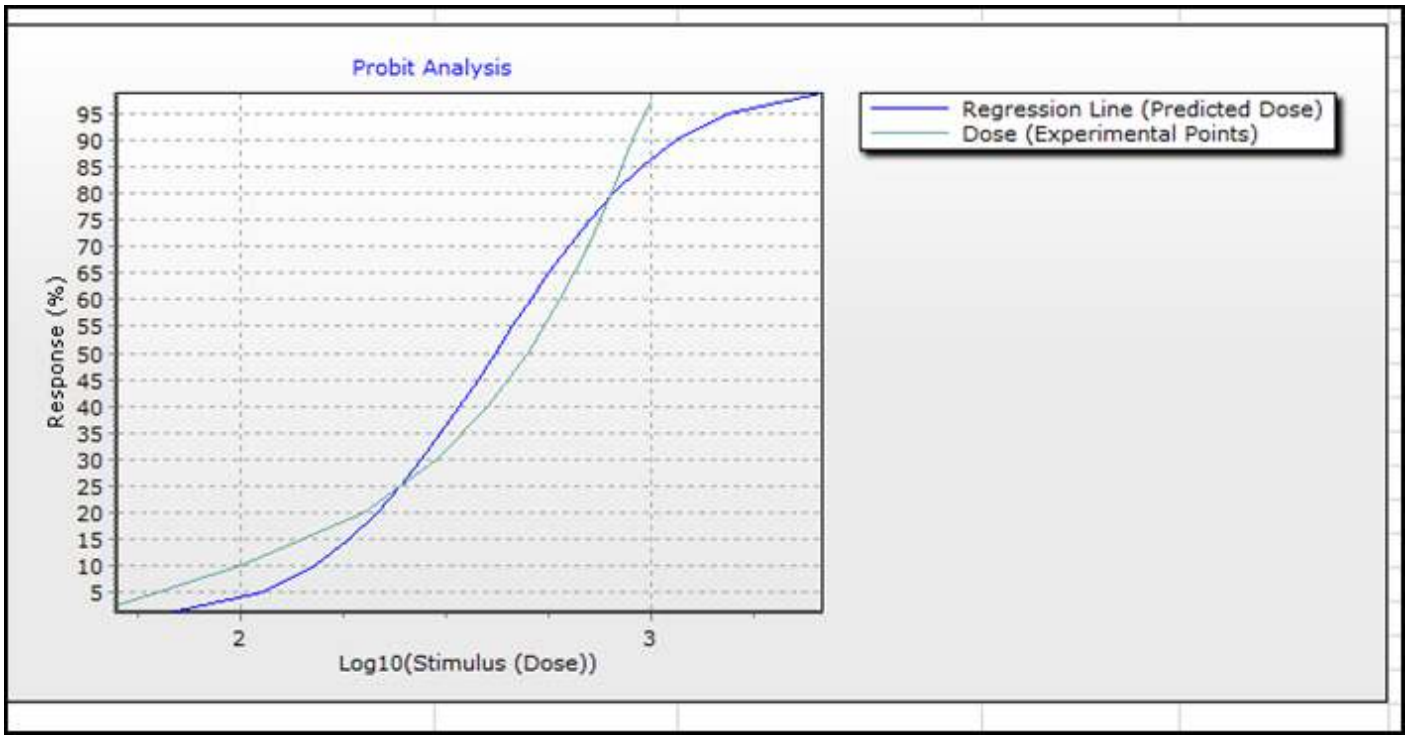


Table 2: The relation between concentration of PE and mortality rate of *O. mossambicus*

Concentration	Actual Percent%	Log10 conc.	Total no.	No. dead	Probit
Control	-----		10	0	-
50	0.025	1.699	10	0	3.0396
100	0.1	2.0	10	1	3.3183
200	0.2	2.301	10	2	4.1585
300	0.3	2.4771	10	3	4.476
400	0.4	2.6021	10	4	4.7471
500	**0.5**	**2.699**	**10**	**5**	**5.0**
600	0.6	2.7782	10	6	5.2529
700	0.7	2.8451	10	7	5.524
800	0.8	2.9031	10	8	5.8415
900	0.9	2.9542	10	9	6.2817
1000	0.975	3.0	10	10	6.9604

Objective 2: To evaluate the Haematological and Biochemical Alterations In*Oreochromis mossambicus* Exposed to Pyrazosulfuron Ethyl.

Overall Experimental setup is presented in Table 3.

Table 3: Experimental Set up:

Chemical used	Treatments						Haematology parameter	Biochemical parameter
Pyrazosulfuron Ethyl (Herbicide)	Group 1: $1/20^{th}LC_{50}$ of PE		Group 2: $1/10^{th}LC_{50}$ of PE		Group 3: $1/5^{th}LC_{50}$ of PE		RBC Hb PCV MCH MCHC MCV WBC	Glucose Protein Albumin Globumin Urea BUN Creatine
	7 Days	14 Days	7 Days	14 Days	7 Days	14 Days		

Sub-lethal Toxicity Test:

Based on LC_{50} value, further sub-acute toxicity studies were conducted in a semi-static condition.Ten fish were exposed to each treatment in a glass aquarium filled with water. A herbicide free group (Control) was maintained simultaneously. The test was renewed at the end of 24 h and freshly prepared herbicide was added to maintain the concentration of PE at a constant level.No mortality was observed in control group during the experiment period. Fishes were being sacrificed on 7^{th} and 14^{th} day and blood was collected for haematological and biochemical analysis.

About 3 - 4ml of blood was collected from the caudal peduncle using heparinized disposable syringe. The blood was stored at -4°C prior to estimation of hematology. Haemoglobin estimation (HB), Pack Cell Volume (PCV), blood glucose level and total serum protein wereanalyzed by NIHON KOHDEN Automated Haematology Analyzer (Celtac alpha, Japan). Red blood cell count (RBC), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC) was be determined using the formulas given below.

$$MCHC = \frac{HB}{PCV} * 1000\ g/dL MCV = PCV * \frac{1000}{RBCs}\ fL MCH = \frac{HB}{RBCs}\ pg$$

Statically analysis was performed using SPSS software (version 16). Data was analysed by one way ANOVA test. Results are presented as Mean ± SEM. Level of significance was set as $p<0.05$, $p<0.01$ and $p<0.001$.

A significant decrease in haematological parameters of all 3 groups expressed a dose and time dependent manner compared to control. A significant decreased in RBC Hb, PCV, MCV, MCH, MCHC and WBC ($p < 0.001$) (Fig 3 to 6).

Several studies have used haematology as a biomarker of pesticide exposure and also to monitor the interaction between a toxicant and biological system (Dacuna*et al*., 2011, Ighwela*et al*., 2012, Desai and Parikh *et al*., 2012 and Parikh *et al*., 2013).Generally, a decrease in nonspecific immunity of the fish due to pesticide exposure leads to alterations in haematological parameter (Svoboda *et al*., 2001). Furthermore, the decrease in the haematological parameter might have resulted from disruptive action of the pesticide on the membranes and cell viability (Hii*et al*., 2007; Koprucu*et al*., 2006). Lysis or shrinkage of erythrocytes due to pesticide action on the erythropoietic tissue may lead to a reduction in

haemoglobin and haematocrit (Saravanan*et al*., 2011). The decrease in RBC count, Hb and PCV in all 3 treated groups indicates a non-specific immunity of the fish to the PE herbicide, as reported by Narra, 2016, who suggest that the decrease in RBC count, Hb and PCV is a response of a defensive reaction of the fish against pesticide stress.

The decrease in MCV and MCH in treated groups may be due to high percentage of immature RBCs in the circulation (Lermen, 2004, Saravanan*et al*., 2011 and Hashemi*et al*., 2017). Furthermore, the observed low concentration of MCHC indicates a decrease in Hb synthesis due to toxic action of PE leading to anaemia (Feiz, 2010) probelly resulting from the stress some bacterial infection (Silveira-Coffigny*et al*., 2004) or due to exposure of agrochemical (Mohammadian*et al*.,2010). The reduction of MCHC in the present study may be due to decrease production of Hb after the exposure of PE (Kazemi*et al*., 2011).Similar results have been obtained by Sarikaya and Yilmaz (2003)for common carp, *Cyprinuscarpio*as well as *Cichlasomadiamerus* (Sattari, 2003).

Changes in serum biochemical parameters can be considered as a suitable factor for detection of sublethal toxic effects on target organs and physiological status of fish exposed to toxin (Adhikari*et al*., 2004).Of major changes in blood biochemical parameters of *O. mossambicus* after exposure to PE. Evaluation of the biochemical parameters in aquatic organisms are widely used to monitor the pollutants and their impact on the health conditions. Moreover, biochemical parameter can serve as marker for toxicant exposure and effects in fish (Vutukuru, 2003). Among the biochemical parameters, plasma glucose and protein are extensively used to assess the stress stimulated by environmental contaminants (El-Sayed*et al*., 2007; Lavanya*et al*., 2011).

Stress condition may elicit rapid secretion of glucocorticoids and catecholamines from adrenal tissue of the fish and the resulting hyperglycaemia has been described as a primary response to most stressors (Pottinger and Carrick., 2001 Ramesh *et al*., 2009 and Khan *et al*., 2016). Hyperglycaemia is a state at which fish respond physiologically to manage the new energetic demand created by the toxicant (Banaee*et al*., (2011) and Dacuna*et al*., (2011). In the present investigation the elevation in plasma glucose level in treated groups can be considered to be manifestation of stress induced by PE and thus can be viewed as a physiological response of the fish to meet critical need for energy for under PE exposure.Fish under stress condition may also mobilize protein to meet energy demand and to maintain increased physiological activity (Martinez *et al*., 2004; Narra, 2016). An increased in glucose

level on exposure of agrochemical may also be due to degranulation and vacuolization of pancreatic alpha cells in the initial stages and demand of beta cells in later stage (Attiai and Badawi*et al*., 2015).Our results are parallel to earlier reported hyperglycemia inNeotropical fish *ProchilodusLineatus* on exposure of glyphosate based herbicide (Langiano and Martinez., 2008), and on common carp *Cyprinuscarpio L*. after acute exposure of atrazine herbicide as an intense metabolic stress response of fish, *O. mossambicus* exposed to fungicide and insecticides (Parikh and Desai., 2016) and by Plant micro nutrient mixture(Sadekarpawarand Parikh 2016).

Total concentration of plasma proteins compared with the base is used as a clinical indicator in measuring the health, stress and body condition of aquatic organisms (Das *et al*., 2004, Toni *et al*., 2013 and Loro*et al*., 2015).The concentration of the fish blood protein serum is an index of general health condition of fish. protein are indispensible constitutes required by organisms in tissue building and play important role on energy metabolism. (Remia*et al.*, 2008, Pang-Hang *et al*.,andParikh and Desai, 2016). In present investigation there was an overall decrease in serum protein contain of treated groups in a dose and time dependent manner. The depletion of protein may be due to increase energy demand leading to increase protein consumption, a process where a protein is converted into energy, and therefore protein serum was reduced. Red blood cells play an important role in oxygen transportation in the body and an insufficient amount of red blood cells has the negative effect on the body of aquatic animals and reduces the total protein in blood plasma. Changes in protein synthesis is one of the most common response to cellular damage, therefore, by measuring the amount of protein it can be correlated with the amount of cell damage (Osman *et al*., 2010). Given that most of the proteins are synthesized in the liver, protein reduction in blood plasma may be related to liver impairment of fish on PE exposure. Our results are in agreement of earlier reported work by Khan *et al*., 2016 and Ramesh *et al*., 2009 on Common carp *cyprinuscarpio* on exposure of herbicide atrazine and glyphosate. As well as on grass carp *Ctenopharhyngodenidella*as reported by Abdali*et al.,* (2011) on exposure of Atrazine.

Serum albumin measures as considerable diagnostic value in laboratory animals because it relates general nutritional status, the integrity of the vascular system and liver function. Albumin in fish participates in plastic metabolism and perform transport functions of substances necessary for life activities (first of all lipids) (Padma Priya*et al*., 2012 and Parikh and Upadhyay *et al*., 2014). Furthermore, serum protein mainly contains albumin and globulin. Albumin is thought to have three basic functions in fish: osmotic regulation of

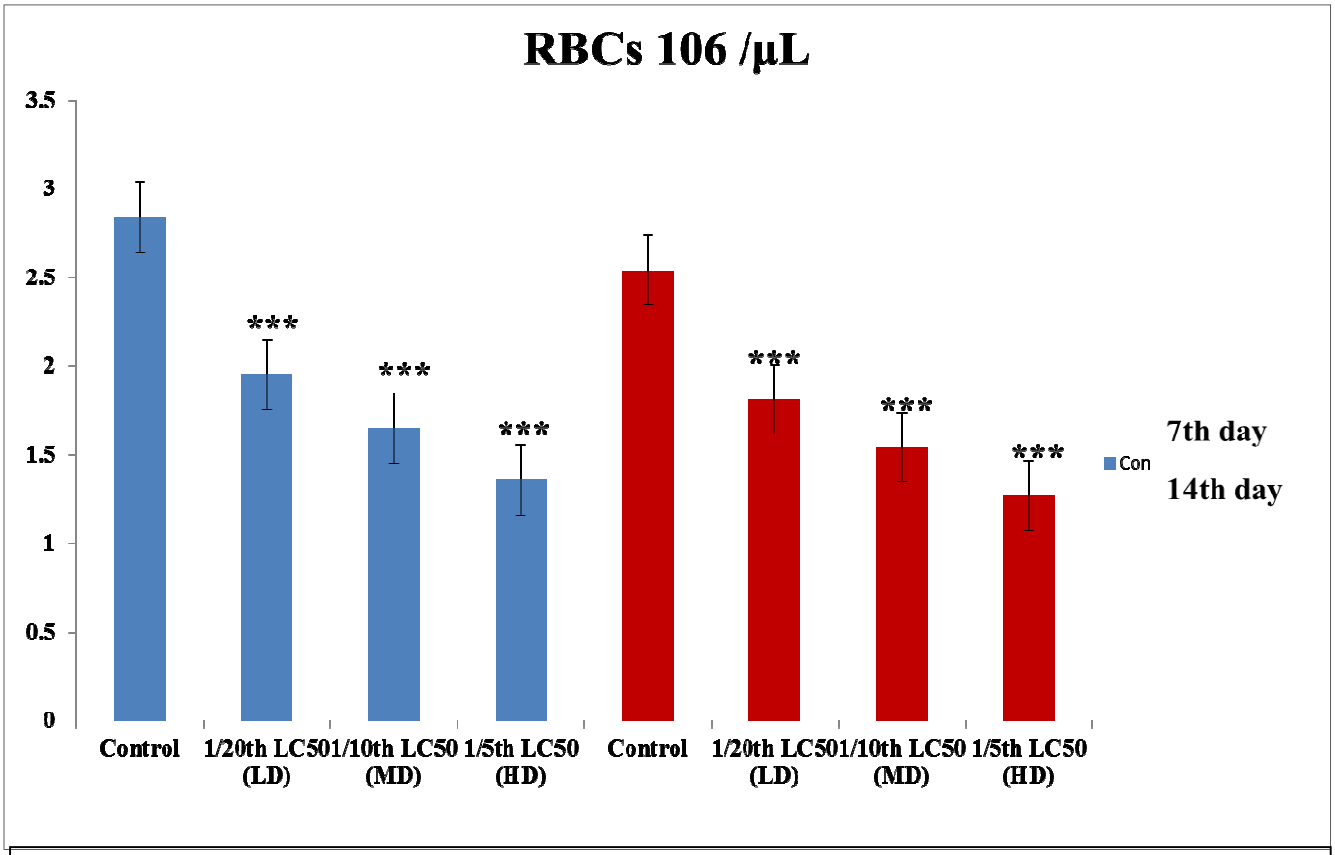
blood volume, source of protein reserve and is also involved in transport functions of exogenous chemicals and endogenous metabolites (Baker, 2002 and Abdali*et al*., 2011). Globulins on the other side are the precursors for the synthesis of immunoglobulins (acting as antibodies) which are the principle components responsible for immunity (Margaret and Robert, 2000).In the present study PE exposure was found to reduced serum albumin and globumin significantly ($p< 0.05$) in dose and time dependent manner. Reduced albumin and globumin ratio is an index used to track changes in composition of serum and is used to predict to liver and kidney dysfunction. Reduced A: G ratio probably indicating the onset of pathological processes (Javed*et al*., 2017).Reduced serum albumin level has been reported in *O. niloticus* on exposure of cypermethrin by El-sayed and Saad (2007) and on exposure of benomyl by Young Min and Kang. In addition Adedeji (2010) and Maheshwari (2010) have reported decreased levels of total protein, albumin and globulin in the African catfish Clariasgariepinus and *Sarotherodon mossambicus* respectively. Thus the reduced globulins reflects the reduction in the immunological defence response due to toxicant, and probably due to utilization of biochemical energy to counteractthe toxic stress caused due to herbicides present in aquatic media (Felix *et al*., 2016).

Urea is a product of the deamination of glucogenicamino acids in the liver usually under conditions that the system requires energy generation to overcome any physiological stressor. Creatinine in serum is a metabolite of muscle Creatinine. The concentrations of urea and creatinine in serum are usually constant because both are easily excreted by the kidneys. Elevated levels of both urea and creatinine therefore, indicate diminished renal function (Jayasundera and Macnab, 2012, Okonkwo*et al*., 2013 and Parikh *et al*., 2013). The dose-dependent elevations in the concentrations of urea and creatinine in the serum of fish exposed to PE are therefore indicative of diminished renal function as a result of kidney damage.In the present study serum Creatinine showed significant increase in treated group in comparison to control group suggesting that there occurs an alteration in glomeruli filtration rate. (El-Bagori., 2001 and Abbass*et al*., 2002).

A major nitrogen-containing metabolic product of protein catabolism is blood urea nitrogen, which acts as a major osmolyte; hence, it can be used as a sentinel tool for gill and kidney dysfunction prediction in fish (McDonald and Grosell, 2006).In the present study significant elevation ($p< 0.001$) in PE exposed fish in comparison to control group may be due to a correlation between urea and increase protein catabolism or from more efficient conversion of ammonia to urea as a result of increased synthesis of enzyme involved in urea production.

Furthermore, the high level of blood urea and Creatinine has either resulted from increased breakdown of tissue / impaired execration / increased synthesis / decreased urinary clearances by the kidney or due to decreased degradation of PE (Adham*et al*., 2002, Amin and Hashem2012 and Parikh *et al*., 2013). Hence the increased serum BUN in PE exposed groups is suggestive of overall disturbance in kidney functions.

Fig 3: Changes in Haemetological Parameter RBC and Hb of PE treated fish O.mossambicus. values are mean ± SE observation 7th and 14th day.



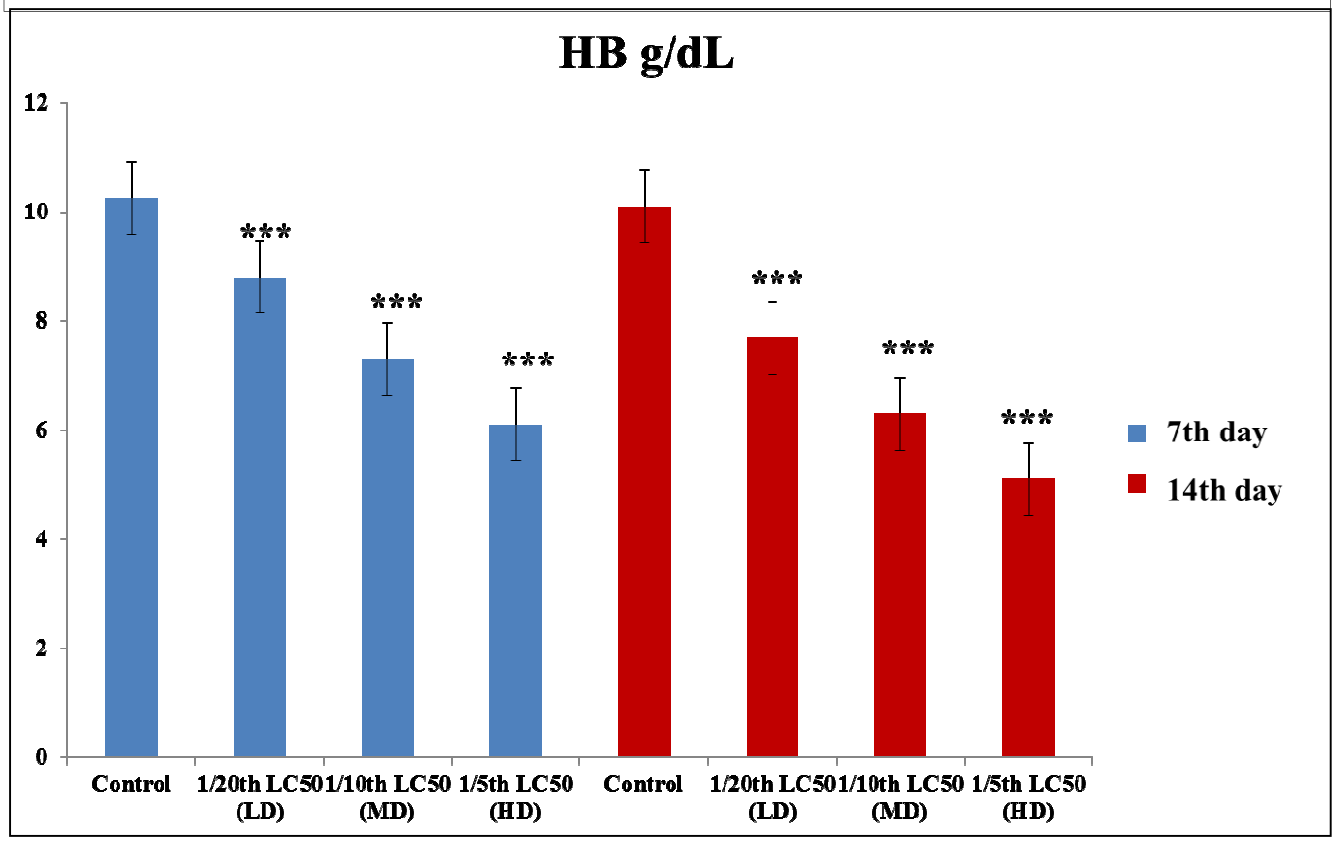


Fig 4: Changes in Haemetological Parameter PCV and MCV of PE treated fish O.mossambicus. values are mean ± SE observation 7th and 14th day.

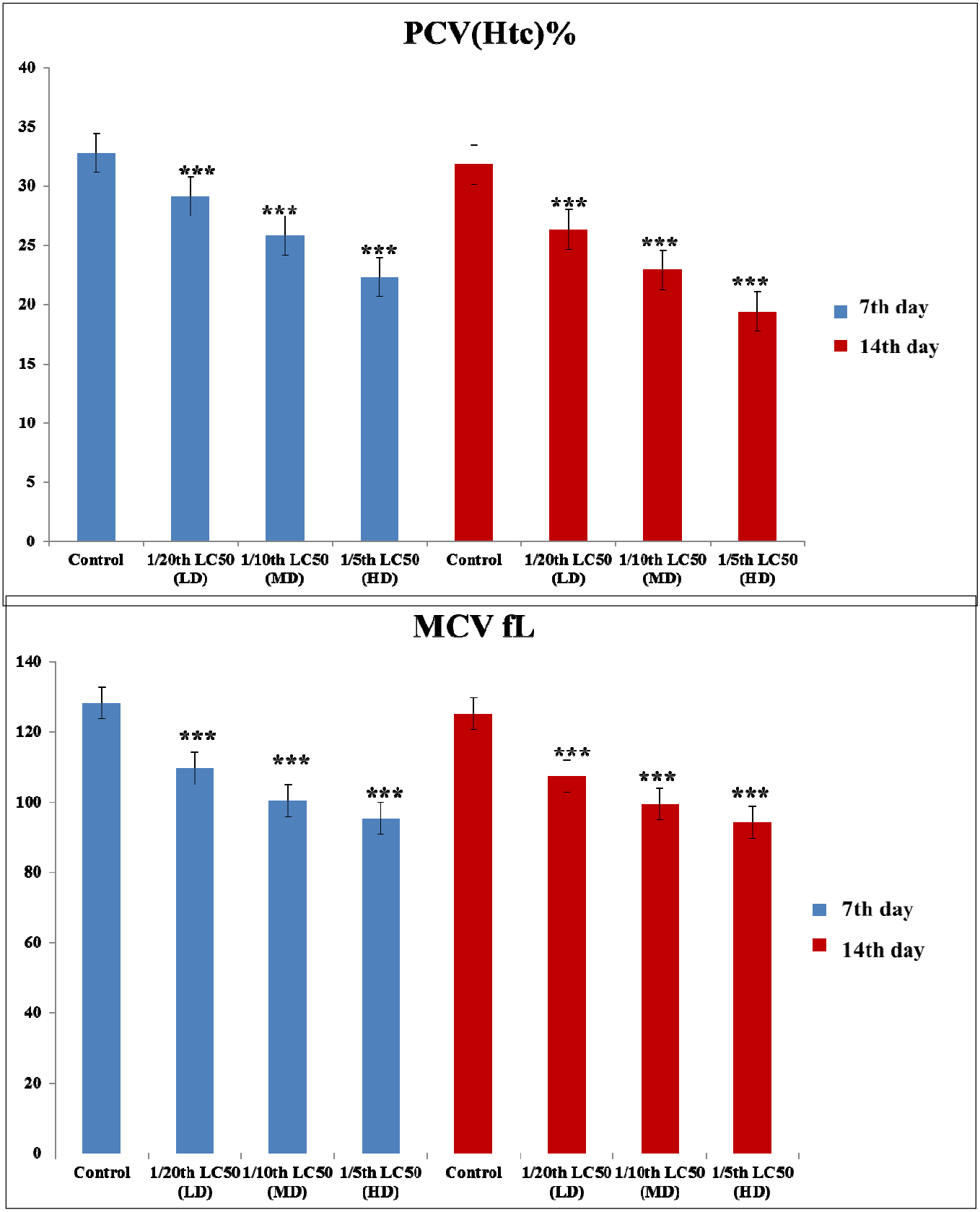
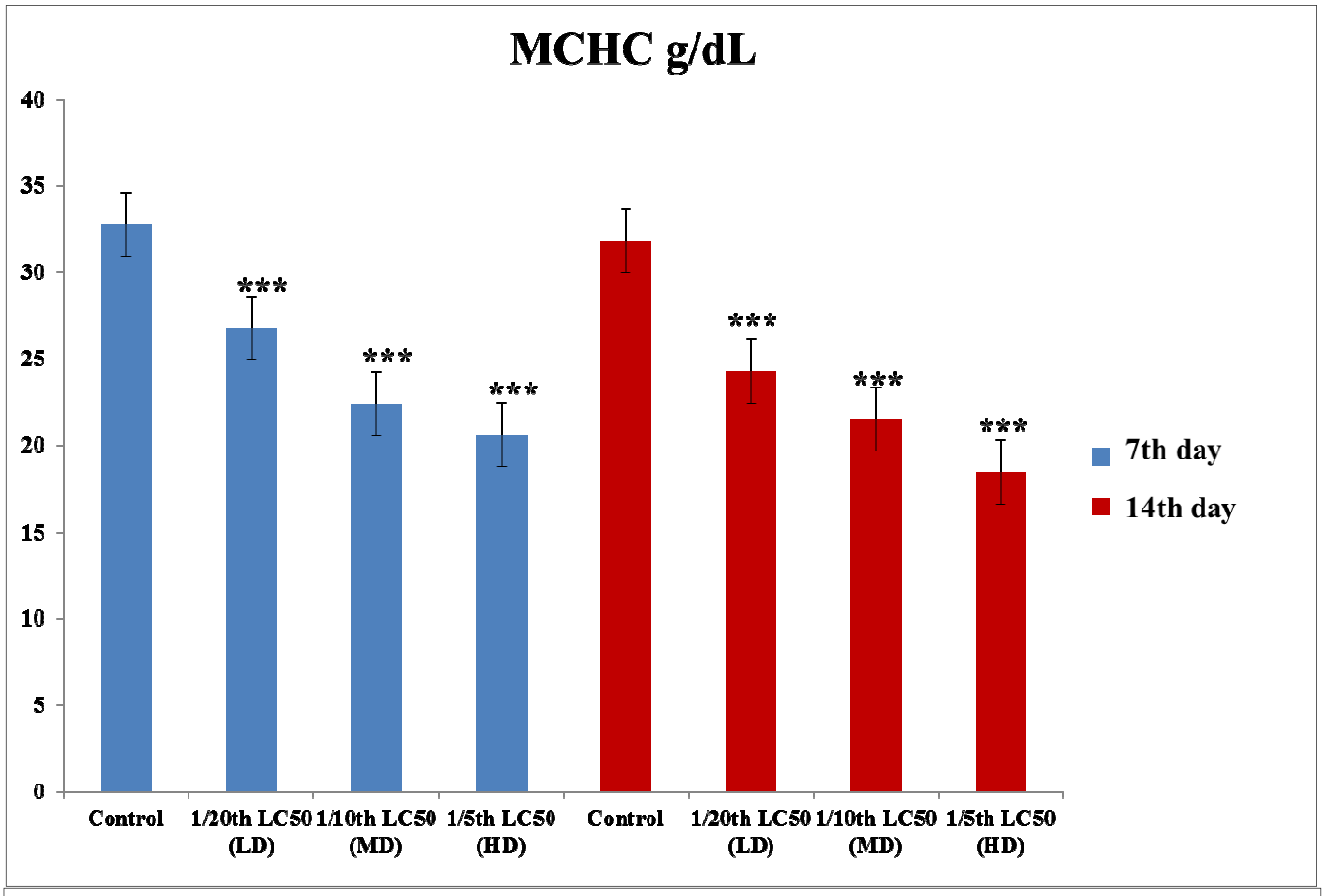


Fig 5: Changes in Haemetological Parameter MCHC and MCH of PE treated fish O.mossambicus. values are mean ± SE observation 7th and 14th day.



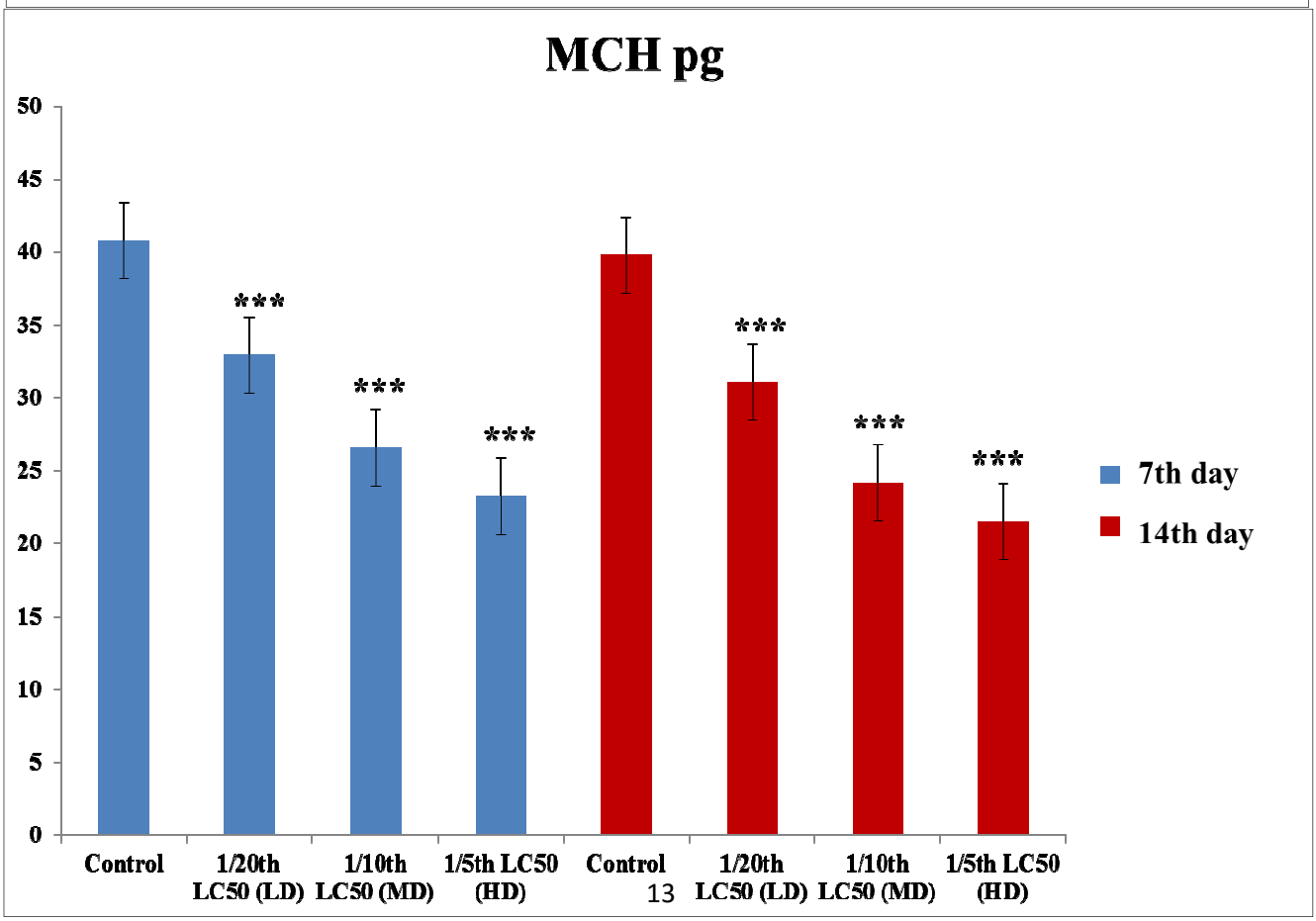


Fig 6: Changes in Haemetological Parameter WBC of PE treated fish O.mossambicus. values are mean ± SE observation 7th and 14th day.

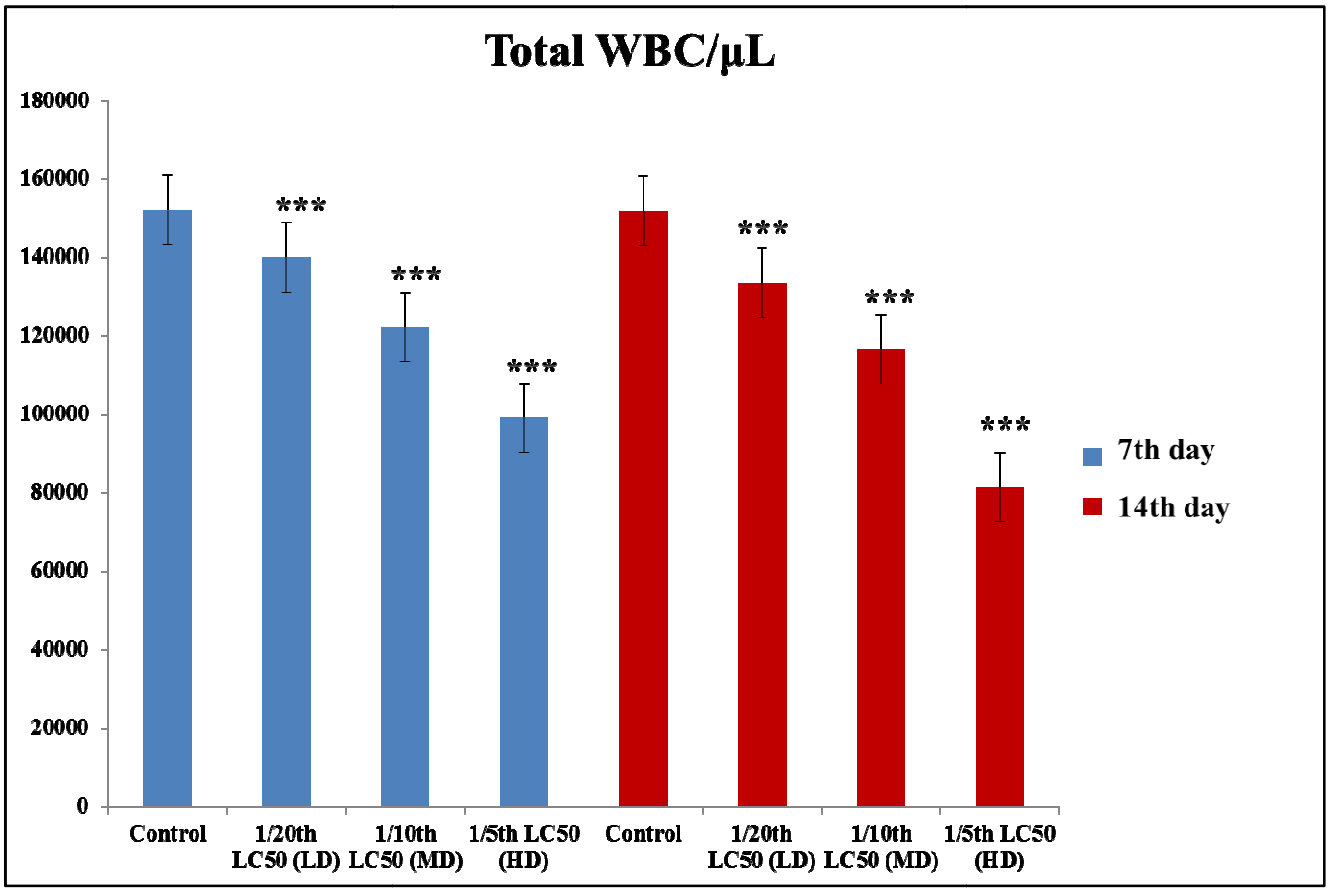
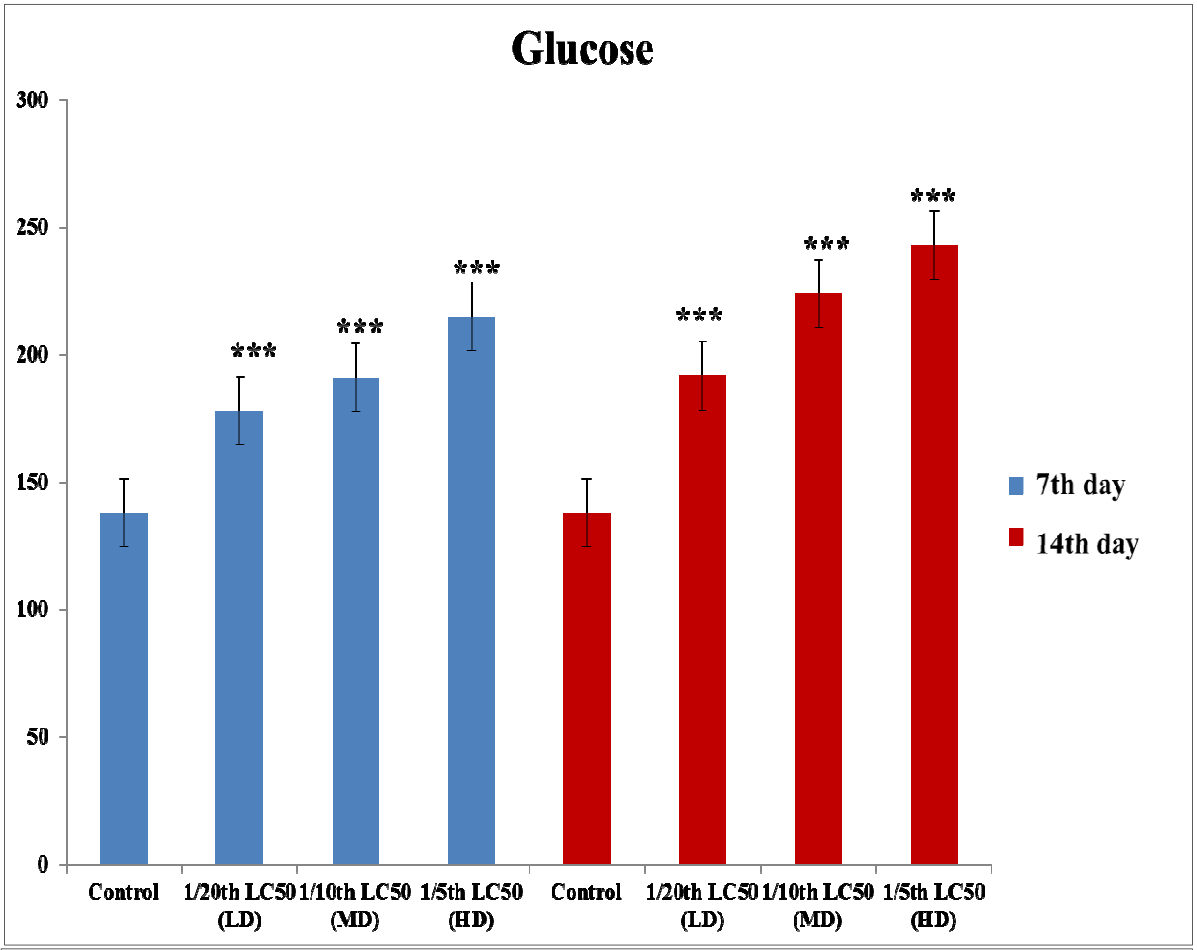


Fig.7: Changes in Biochemical Parameter Glucose and Total Serum Protein of PE treated fish *O.mossambicus*7thand14th day.



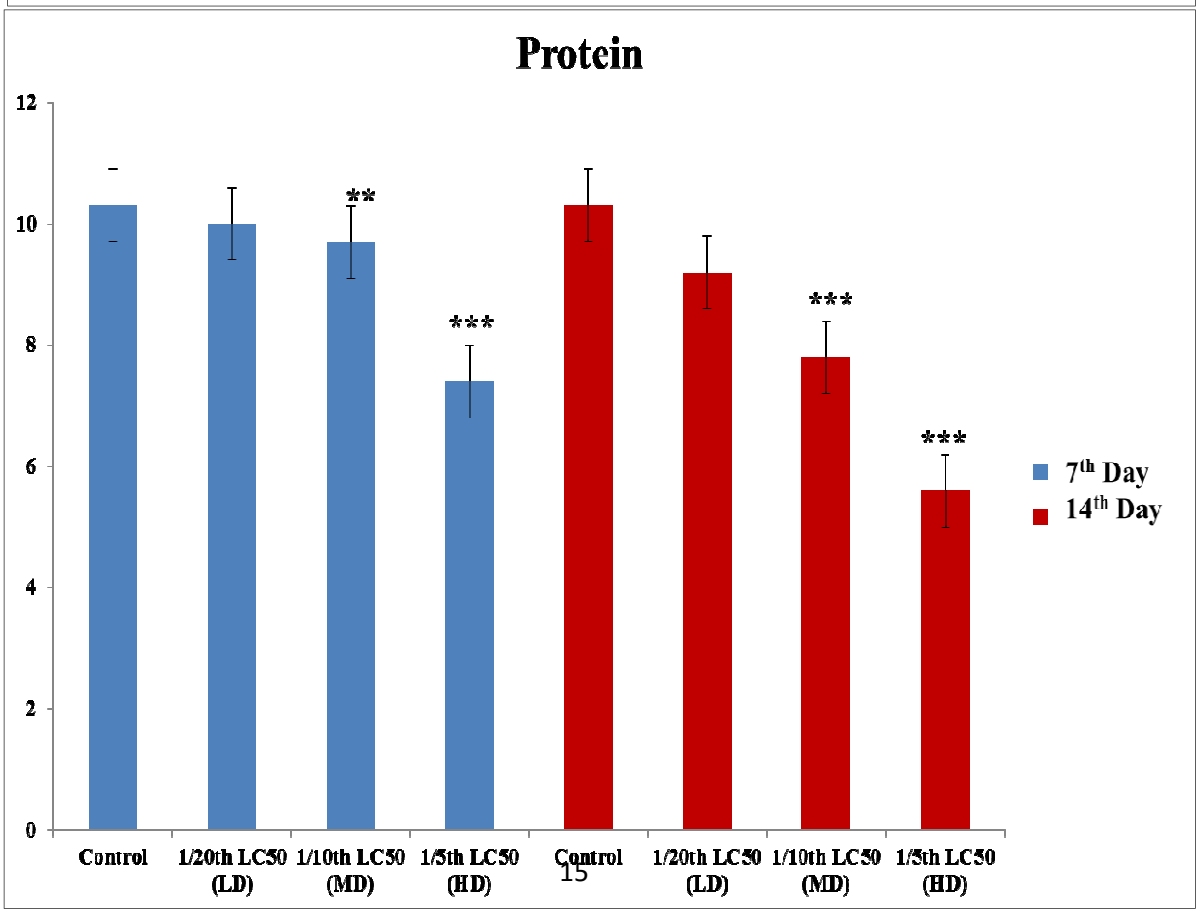
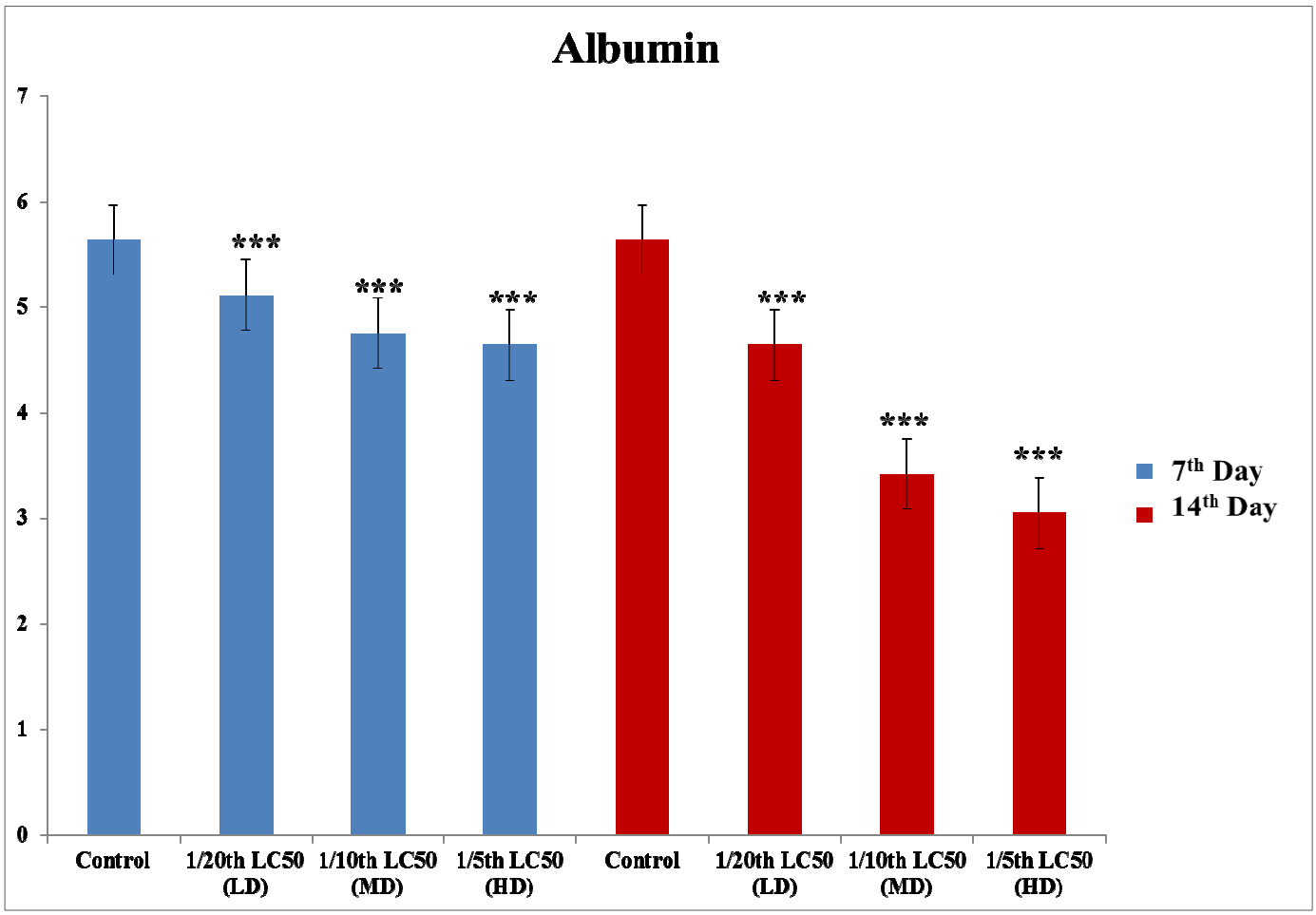


Fig.8: Changes in Biochemical Parameter Serum Albumin and Globumin of PE treated fish *O.mossambicus*7th and 14th day.



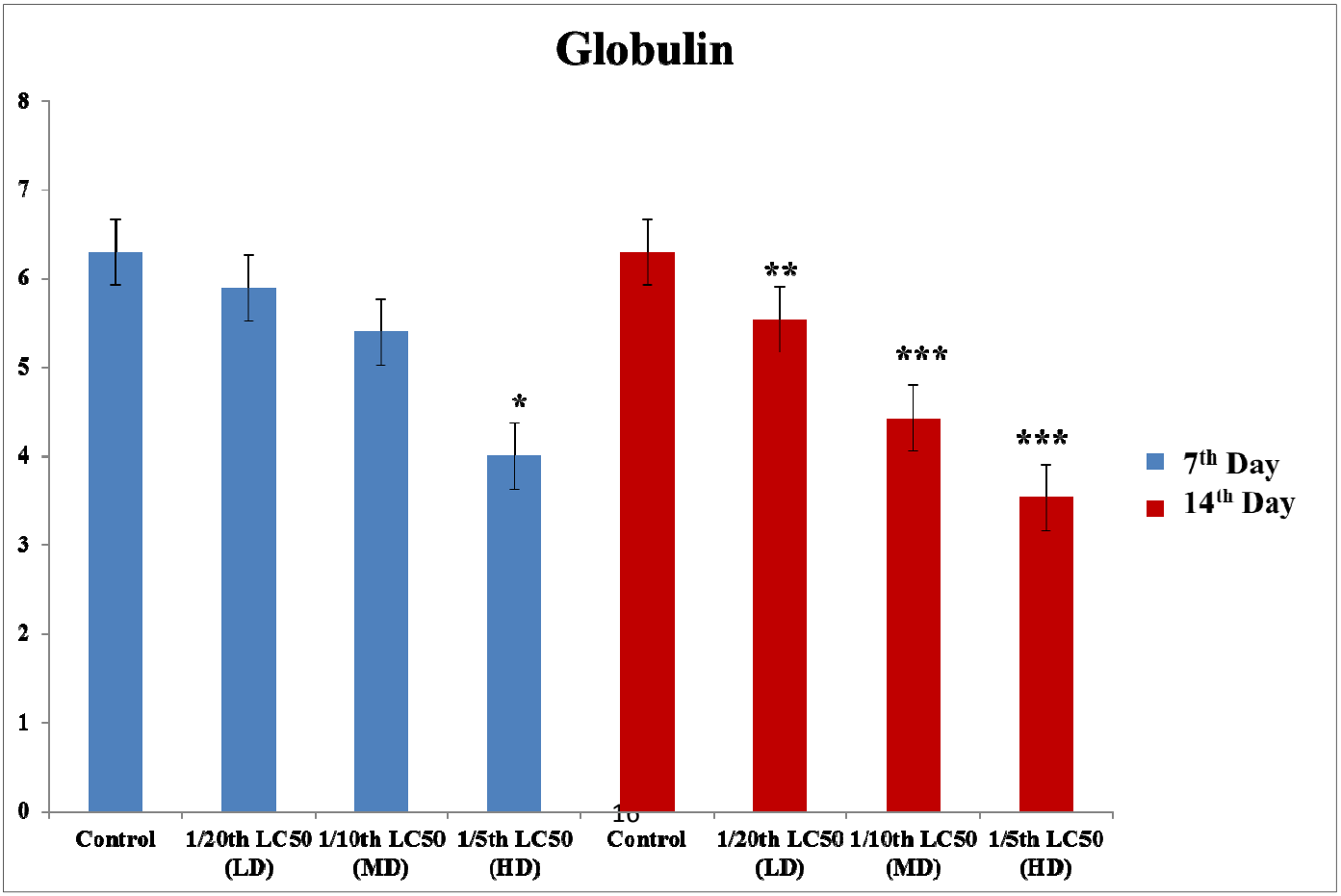
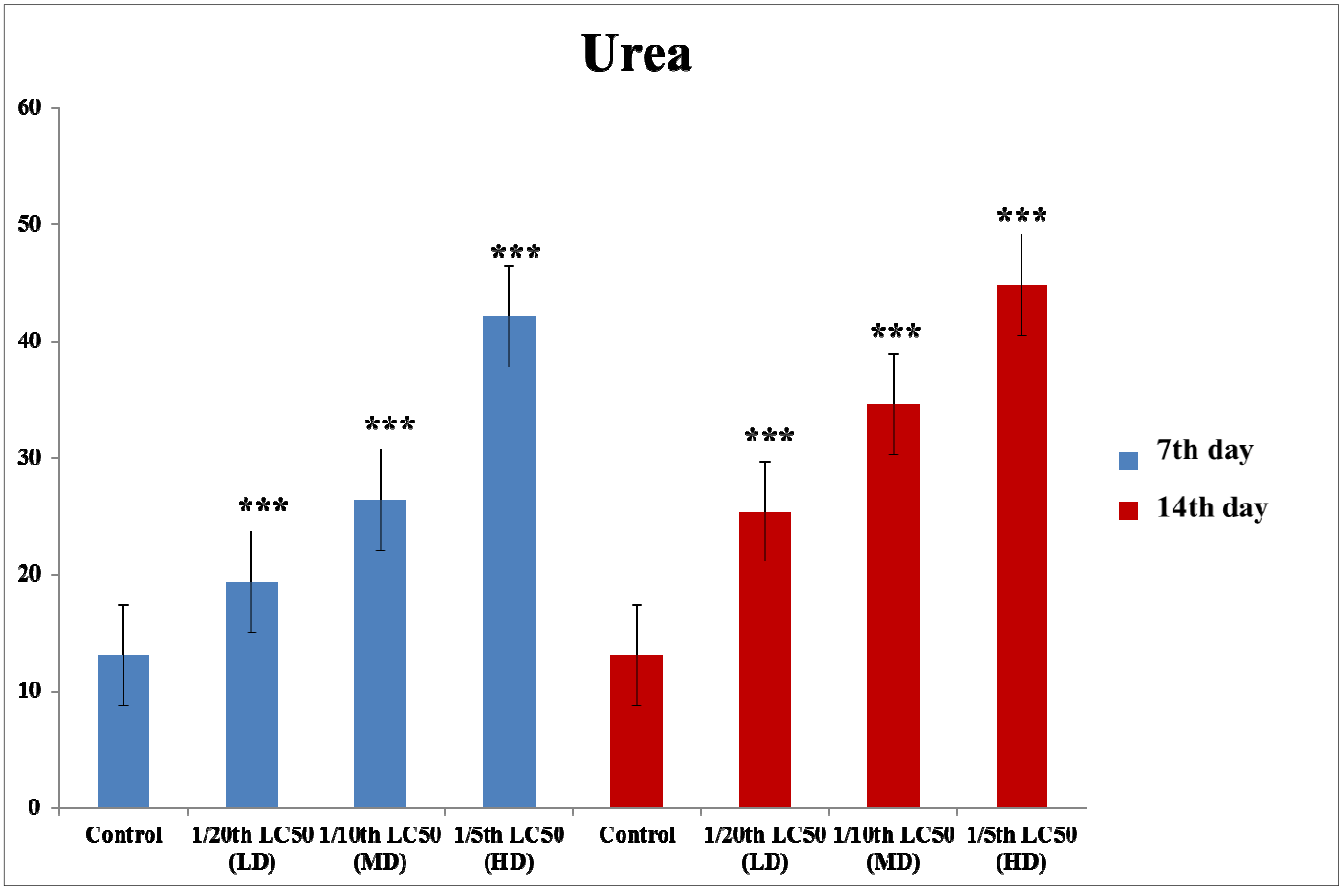


Fig.9: Changes in Biochemical Parameter Blood Urea and BUN of PE treated fish *O.mossambicus*7th and 14th day.



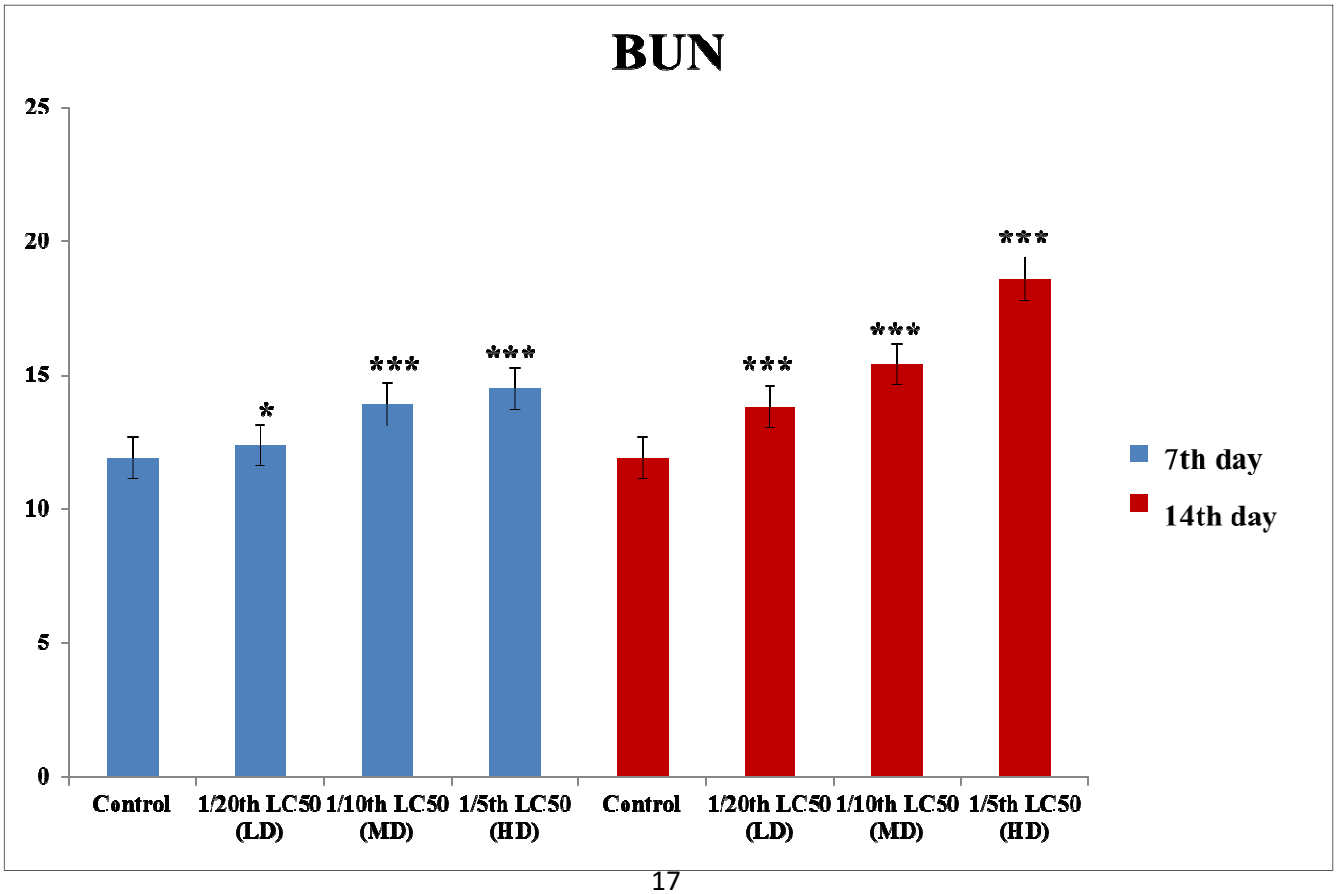
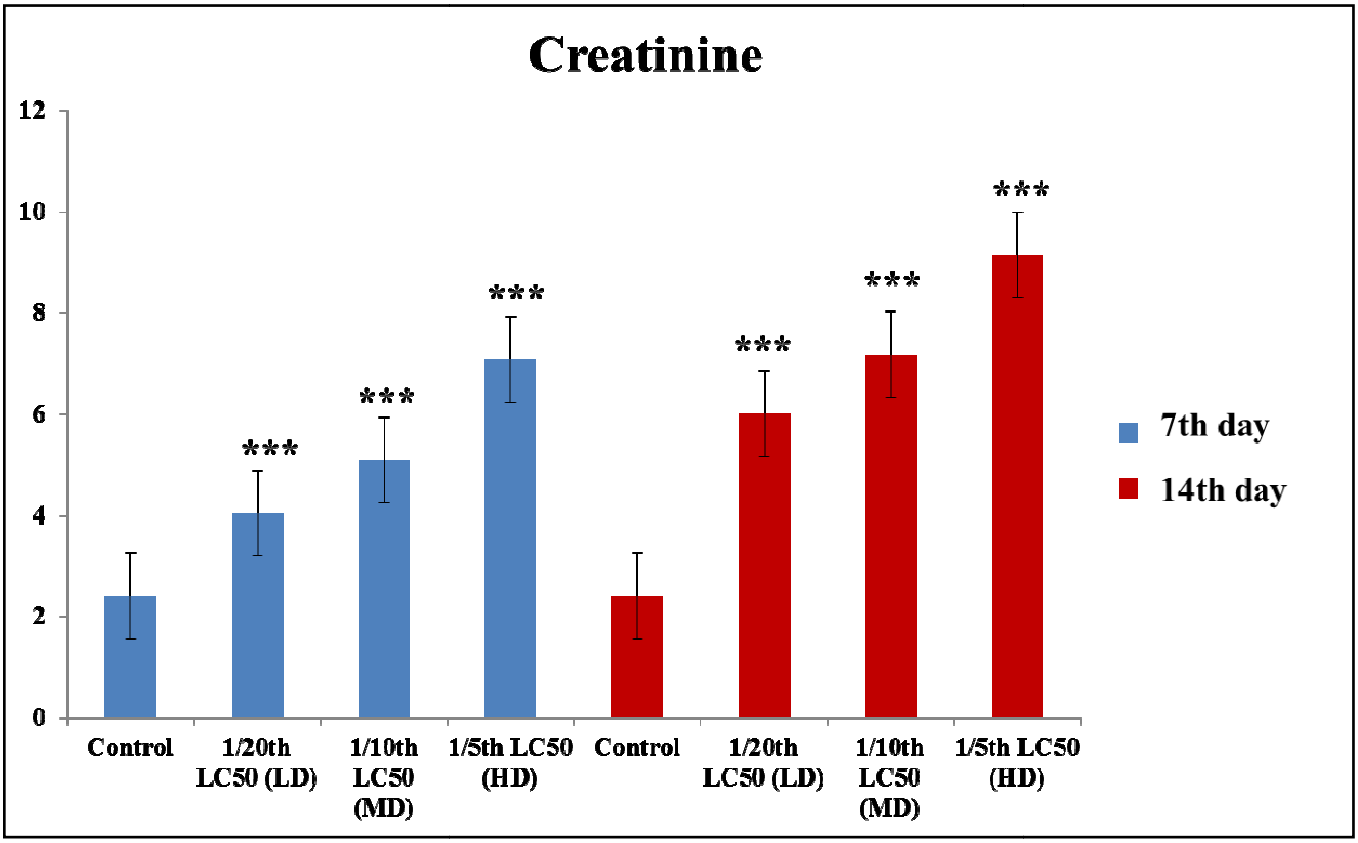


Fig.10: Changes in Biochemical Changes Creatinine of PE treated fish *O.mossambicus*7th and 14th day.



<u>Objective 3 :</u>To determine the health status of *O.mossambicus* exposed to Pyrazosulfuron Ethyl using a histology-based health assessment protocol (Smith , 2012).

After blood collection fish were sacrificed and macroscopic alternations in internal organs were noted. The variable used for gills was: whether the gills were normal, frayed, and pale or clubbed. Liver was considered normal if it red or pale red, presence or nodules and focal discolouration was considered abnormal. Kidney was graded as normal or mottled, mottled is the patchy condition which ranged from scattered patches of grey to total grey discoloration. Fishes were being sacrificed on 7^{th} and 14^{th} day and after decapitation, the blood wasbeing drained out and the fishes were dissected for the histology of Gills, Liver and Kidney. Fresh tissues were fixed in 4% paraformaldehyde for 24 hours, dehydrated, embedded in paraffin wax and sectioned at 10-12µm then stained with haematoxylin and eosin and examined microscopically and photographed using digital camera (400x).

Histology assessment:

The histology assessment was done by applying the histopathological tool as proposed by van dyk*et al*., (2006) applying the histopathological tool, histological results can be expressed qualitatively and quantitatively, allowing for statical comparison between treated and control group without discarding qualitative results. Incorporation of quantitative methods is essential to the continued development of histopathological indices of pollution exposure, and to the interpretation of histological responses (Pieterse 2008). Further as processed by Barnet *et al.,* 2004, histopathological assessment protocol can leads to standardized quantification allowing the comparison and have been applied in various studies (Schmidt-posthaus*et al.,* 2001, Van dyk*et al.,* 2006 and Smith 2012).

Microscope slides were assessed qualitatively to identify histological alterations and were compared with control fish.Following the reaction pattern as described by Pieterse *et al*., 2010 the histological alterations were assessed for Circulatory disturbance (CD), Regressive changes (RC), Progressive changes (PC) and Inflammation (inf). Based on the qualitative observations in the form of reaction pattern semi-quantitative assessment was done according to the protocol of Van Dyk*et al*., (2009).

The organ index is an indication of the number and severity of histological alterations in a particular organ of a species. The organ indices were classed according to the classes of Van Dyk*et al*., (2009) with Class 1 (index<10) being tissue with slight histological alterations; Class 2 (index 10-25) being tissue with moderate histological alterations, Class 3 (index 26-

35) being tissue with pronounced alterations whereas Class 4 (index >35) being tissue with severe alterations. The fish index is a sum of all of the organ indices for any given fish sampled.

An importance factor (W) ranging from 1 to 3 was assigned to each alteration according to Smith (2012). Based on the histological alterations were described into three importance factors (W)

(1) Minimal pathological importance, the lesion is easily reversible as exposure to irritant ends;

(2) Moderate pathological importance, the lesion is reversible in most cases if the stressor is neutralised;

(3) Marked pathological importance, the lesion is generally irreversible, leading to partial or total loss of organ function. Further,each alteration was assigned a score value (a) from 0 to 6, depending on the degree and extend of alteration.

(0) unchanged,

(2) mild occurrence,

(4) moderate occurrence that is alteration present in half of the tissue

(6) severe alteration that is present all over the tissue.

Reaction index of an organ (Iorg rp) was calculated using the formula:

$$\boldsymbol{Iorg\ rp = \sum alt\ (a\ orgrp\ alt\ \times W\ org\ rp\ alt}$$

Where: org, rp = constant. This index is calculated by the sum of the multiplied importance factors and score values of the alterations of the corresponding reaction pattern.

Organ index (I org) was calculated using the formula:

$$\boldsymbol{Iorg = \sum rp \sum alt\ (a\ org\ rp\ alt\ \times W\ org\ rp\ alt}$$

Where: org = organ (constant); rp = reaction pattern; alt = alteration; a = score value; w = importance factor. This index is the sum of the multiplied importance factors and score values of all changes found within the assessed organ.

The reaction index (I org rp) gives an indication of the quality of the lesions in the specific organ, and the organ index (Iorg) gives an indication to the degree of damage.Table 4 and 5 summarizes the quantitative results obtained after the exposure of PE for 7 and 14 days at high, medium and low doses.

Time dependent and dose dependent an alteration in the reaction pattern was obtained for Gills, Liver and Kidney.

Gills: As far as gills are concerned the circulatory disturbance, regressive changes were comparatively higher only after 14th day of exposure. The progressive changes were not that significant. The organ index (I org) of gillsat low dose was 0 followed by 7 at the medium dose and 9 at high dose after 7th days of exposure, signifying that the gills tissues showed slight histological alterations. After 14th day the low dose of PE exposure gill organ index (I org) was 14 followed by 27 at medium dose and 38 at high dose, signifying that the gill tissue had moderate, pronounced to severe alterations. Our results are in agreement with work of Van Dyk et al., 2009 who have shown altered necropsy based health assessment index.

Liver: The organ index (I org) of liver at low dose was 5 followed by 10 at medium dose and 13 at high dose after 7th days of PE exposure, signifying that the liver tissues showed slight to moderate histological alterations. After 14th day the low dose of PE exposure liver organ index (I org) was 21 which comes under moderate alteration suggesting that the lesions can be reversible if the fishes were allowed for recovery (Ackermann., 2008). At medium dosethe liver organ index was 31 followed by 43 at high dose with pronounced and severed histological alterations respectively, suggesting that these alterations will be difficult to get back to normal even after recovery (Smith, 2012)

Kidney: The organ index (I org) of kidney at low dose was 5 followed by 8 at medium dose and 10 at high dose after 7th days of exposure, signifying that the kidney tissues showed slight to moderate histological alterations. After 14th day the low dose of PE exposure kidney organ index (I org) was 18 at low dose followed by 31 at medium dose and 43 at high dose, signifying that the kidney tissue had moderate, pronounced to severe alterations which are irreversible changes probably leading to partial or total loss of organ function(Ackermann., 2008).

Table 4: Summary of the reaction index (Iorg rp), Organ index (I org), Total reaction index (I rp) and the total index (Tot-I) of the selected organ as calculated for the groups $1/20^{th}$ LC_{50}, $1/10^{th}$ LC_{50} and$1/5^{th}$ LC_{50} for the 7^{th} days exposure of PE. (CD-circulatory disturbance, RC-regressive changes, PC-progressive changes, I- Inflammation)

	Gill			Liver			Kidney		
Reaction pattern	**$1/20^{th}$ LC_{50}**	**$1/10^{th}$ LC_{50}**	**$1/5^{th}$ LC_{50}**	**$1/20^{th}$ LC_{50}**	**$1/10^{th}$ LC_{50}**	**$1/5^{th}$ LC_{50}**	**$1/20^{th}$ LC_{50}**	**$1/10^{th}$ LC_{50}**	**$1/5^{th}$ LC_{50}**
Iorg rp									
CD	0	2	2	0	1	1	1	1	2
RC	0	3	5	5	9	10	3	5	6
PC	0	2	2	0	0	2	2	2	2
Iorg	**0**	**7**	**9**	**5**	**10**	**13**	**5**	**8**	**10**

Table 5: Summary of the reaction index (Iorg rp), Organ index (I org), Total reaction index (I rp) and the total index (Tot-I) of the selected organ as calculated for the groups $1/20^{th}$ LC_{50}, $1/10^{th}$ LC_{50} and$1/5^{th}$ LC_{50} for the 14^{th} days exposure of PE. (CD-circulatory disturbance, RC-regressive changes, PC-progressive changes, I- Inflammation)

	Gill			Liver			Kidney		
Reaction pattern	**$1/20^{th}$ LC_{50}**	**$1/10^{th}LC_{50}$**	**$1/5^{th}$ LC_{50}**	**$1/20^{th}$ LC_{50}**	**$1/10^{th}$ LC_{50}**	**$1/5^{th}$ LC_{50}**	**$1/20^{th}$ LC_{50}**	**$1/10^{th}$ LC_{50}**	**$1/5^{th}$ LC_{50}**
Iorg rp									
CD	3	4	6	1	2	2	2	4	6
RC	9	20	24	16	24	32	14	25	32
PC	2	3	8	4	5	9	2	2	5
Iorg	**14**	**27**	**38**	**21**	**31**	**43**	**18**	**31**	**43**

A result of the present investigation indicates that administration of sub-lethal concentration of PE is toxic to O.mossambicus and has resulted intosignificant alterations in haematological, biochemical and histological parameters.These alterations may provide an early warning signal for the determination of toxic level of herbicide and their effects in aquatic organisms.The herbicide therefore can be classified as toxic for fish. However the genotoxic studies and field based study will validate the toxic potential of PE on non-target organisms present in that ecosystem.