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Effect of 2,4 Dichlorophenoxy Acetic Acid (Herbicide) on the Haematological and Histopathological Parameters of Freshwater Fish *Oreochromis mossambicus*

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Abstract: The tilapia fish *Oreochromis mossambicus* was used to evaluate the sublethal toxic effects of herbicide, 2,4 Dichlorophenoxy acetic acid. Fish were exposed to sublethal concentration (40 mg/l) of 2,4 D for 30 days. The control group was also run simultaneously without any treatment. Haematological parameters such as RBC count, Hematocrit indices and platelet count were decreased while leucocyte count increased significantly. The significant decrease in erythrocyte count indicates osmoregulatory dysfunction, reduction of erythropoietic activity and anemic response. The reduction of hematocrit indices indicate worsening of organisms state, less oxygen carrying capacity, hypochronic microlytic anemia. Increase in WBC count indicates the induced proliferation, and increased antibody production for survival and recovery of the fish exposed to sublethal concentration of herbicide. Histological examination revealed that 2,4 D exposure caused degeneration and congestion, hyperplasia in gills. Liver cells showed extensive fatty degeneration and congestion in liver parenchyma compared to control tissues. Thus, the present study clearly demonstrates that exposure to sublethal concentration of 2,4 D to fish may provoke an adverse effects on vital organs.

Keywords: 2,4 Dichlorophenoxy acetic acid, *Oreochromis mossambicus*, Acute toxicity, Haematology, Gill, Liver, Histopathology

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Introduction

It recent times, pollution of water resources has become a major problem which leads to several ecological disorders and many physiological as well as biological changes in the aquatic organisms. Freshwater fishes are important for our health as they provide nourishment in the form of proteins. It plays a major role in nutrient cycle about because it store large proportion of ecosystem nutrients in their tissues. About 94% of all freshwater fisheries occur in developing

countries (FAO, 2007). Primarily freshwater fishes provide nutrition to the poor people's basket and also livelihood for millions of poorest people.

Chemicals are used extensively in current farming operations (Farah *et al.,* 2004). Agricultural insecticides used to control pests, unwanted herbs, and agricultural illnesses have been discovered to have negative environmental consequences (Sarikaya and Yilmaz, 2003). In freshwater waterways and agricultural areas around the world, herbicides are employed to reduce nuisance and invasive plant species (Baharuddin *et al.,* 2011; Harrahy, Edwards and Hedman, 2014; Baumgartner *et al.,* 2017). Herbicides can enter surface waters through a variety of routes, with runoff and drainage from agricultural areas being the most common (Knauer, 2016). Wastewater treatment plants, storm sewers or combined sewer overflows, and runoff from metropolitan areas are all sources of herbicide pollution in surface water (Wittmer *et al*., 2010; Ensminger *et al.,* 2013).

The majority of pollution of aquatic life occurs on land. Agricultural overspill and waste products carried by the wind, as well as water overflow, are the main causes. Herbicides used on crop areas have contaminated or combined with freshwater bodies with fish near paddy fields. Herbicides are exposed to fish in water bodies surrounding paddy fields in three ways-- dermally (direct absorption through the skin by swimming in herbicide), breathing (direct absorption of herbicide via the gills during respiration), and orally (drinking water which containing herbicide) (Louis *et al.*, 2019).

Herbicides have a variety of effects on organisms. The rate of accumulation of these compounds on biota is dependent on the type of related food chain, as well as the herbicide's physicochemical qualities (chemical stability, solubility, photo-decomposition, and soil sorption) (Rand and Petrocelli, 1985). Herbicide pollution of aquatic ecosystems has been described as a serious global concern. The usage of these compounds in the control of aquatic vegetation, leachate, and runoff from agricultural regions has contaminated the water (Ying and Williams, 2000).

The study of the effects of environmental toxins on aquatic creatures is known as aquatic toxicology (Louis *et al.*, 2009). To evaluate the dangers posed to the aquatic environment, aquatic toxicology entails measuring pollutant levels and assessing harm to freshwater and marine organisms. This branch of study also contains information on how potential risks in and near aquatic habitats can influence humans (Samantha *et al.,* 2020).

One method commonly used to assess fish physiological status and health is haematological examination (Docan *et al.,* 2018). Because blood is the pathophysiological indicator of the entire body, haematological parameters are critical in determining the structural and functional rank of a toxicant-treated animal (Jenkins *et al.,* 2003). The use of haematological status in diagnosing fish welfare and diseases is only possible if information about the ranges of physiological variations of haematological parameters is available, as well as knowledge about the cause and effect, relationship between changes in the external and internal environment and changes in the fish blood picture. All fish show common ecophysiological phenomena that greatly influence hematological status (Ivanc *et al.,* 2005).

Histology is an important tool in fish disease diagnosis because it allows the comparison of normal tissue structures or morphology to that of diseased fish. However, accurate diagnosis and confirmation of disease-related changes necessitate proper specimen processing and a high level of expertise in histopathology (Elena *et al.,* 2010).

The present study was aimed to evaluate the effects of 2,4 dichlorophenoxy acetic acid on the growth and health of the fish *Oreochromis mossambicus*. The current study's specific objectives were to determine the lethal concentration (LC_{50}), investigate the haemato-

logical parameters (RBC, WBC, Hb, PCV, MCH, MCV, MCHC, and Platelet count), and histology of vital tissues such as gills and liver of *Oreochromis mossambicus* exposed to 2,4 Dichlorophenoxy acetic acid.

Materials and Methods

Test animal:

The tilapia fish, *Oreochromis mossambicus* (10-12 cm), was caught from a local fish farm in Thrissur, Kerala, India. Oxygenated tubs were used to transport the fish to the lab. The fish were acclimatized for two days in laboratory's storage tank. The fish were fed commercial fish pellets during acclimatization.

Test Chemical:

In this study 2,4 Dichlorophenoxyacetic acid was purchased from commercial store and used for exposure to the fish. It is commonly known by the ISO code 2,4D. It is a systematic herbicide that kills the majority of broadleaf weeds in paddy fields. It is the most commonly used herbicide in the Palakkad district, where it is applied to a variety of crops sites. *Experimental design and Treatment*:

The fish were acclimatised for 2 days in the laboratory fish storage tank. They were fed commercial synthetic feed (pellets) twice a day. The fish *Oreochromis mossambicus* (10-12cm) were randomly distributed in 15 L plastic tubs. One tub was employed as control, while the others were given different concentrations of 2, 4 D (100 mg/l, 200 mg/l, 300 mg/l, 400mg/l 500mg/l and 600mg/l) to find out the LC_{50}. Six fish were used in each tub for each concentration of herbicide 2, 4-D, and the mortality was recorded after 24, 48, 72, and 96 h. The experiment was designed to expose the fish to separate tubs containing different sublethal concentrations (40 mg/l, 60 mg/l, 80 mg/l and 100 mg/l) of 2,4 D. Each tub contained six fish. The experiment lasted 30 days. During the experiment, the fish were fed artificial food twice a day. Each day, the tubs were refilled with fresh water, and the appropriate concentration of 2,4 D was induced in the tubs.

Fish were starved for 24 h after the 30 day treatment. The blood specimen was collected. Each fish was gently caught with a dip net and blood was drawn from the common cardinal vein with sterilized insulin needles and collected in EDTA-coated eppendorf tubes. Blood samples (control and experimental groups) were immediately used to determine haematological parameters such as total erythrocyte, leucocyte count, haemoglobin (Schaperculaus, 1991), red blood cell indices, and platelet count (Dacie Lewis, 2001).

After collection of blood, gills and liver were removed from both the control and experimental groups after 30 days and preserved in 10% buffered formalin for histological studies.

Statistical analysis:

The dose responses of mortality were studied using a computer-based probit analysis method. Using the statistical programme for social sciences (SPSS) software, version 2, the statistical difference between test groups was calculated using analysis of variance (one-way ANOVA). The data is presented as the mean±standard error for six animals per group.

Results

Acute toxicity:

The results of Probit analysis are shown in the Table 1. In the present study, 50% mortality was observed at a concentration of 400 mg/l of 2, 4 - Dichlorophenoxy acetic acid. The log concentration using Probit analysis (Finney, 1953) is 5.972. The corrected mortality data were analyzed by following the method of Finney (1957) to determine the LC_{50} values. Figure 1 represents the plot of probability (of mortality) of estimated concentration of 2,4-D which indicates the 96 h LC_{50} as 392.17 mg/l of 2,4 D for *Oreochromis mossambicus.*

Effect of 2, 4 D on haematological parameters: The results of haematological examination of blood samples from acute toxicity test are given in Table 2. It is evident that the acute exposure to

Table 1: LC_{50} value of 2,4 Dichlorophenoxy acetic acid and the 95% confidence limit in *Oreochromis mossambicus*

LC_{50} log Concentration	95% confidence		**Probit equation**	**Chi-square**
	Lower limit	**Upper limit**		
5.972	5.887	6.027	Y = -53.613 + 8.978x	1.362

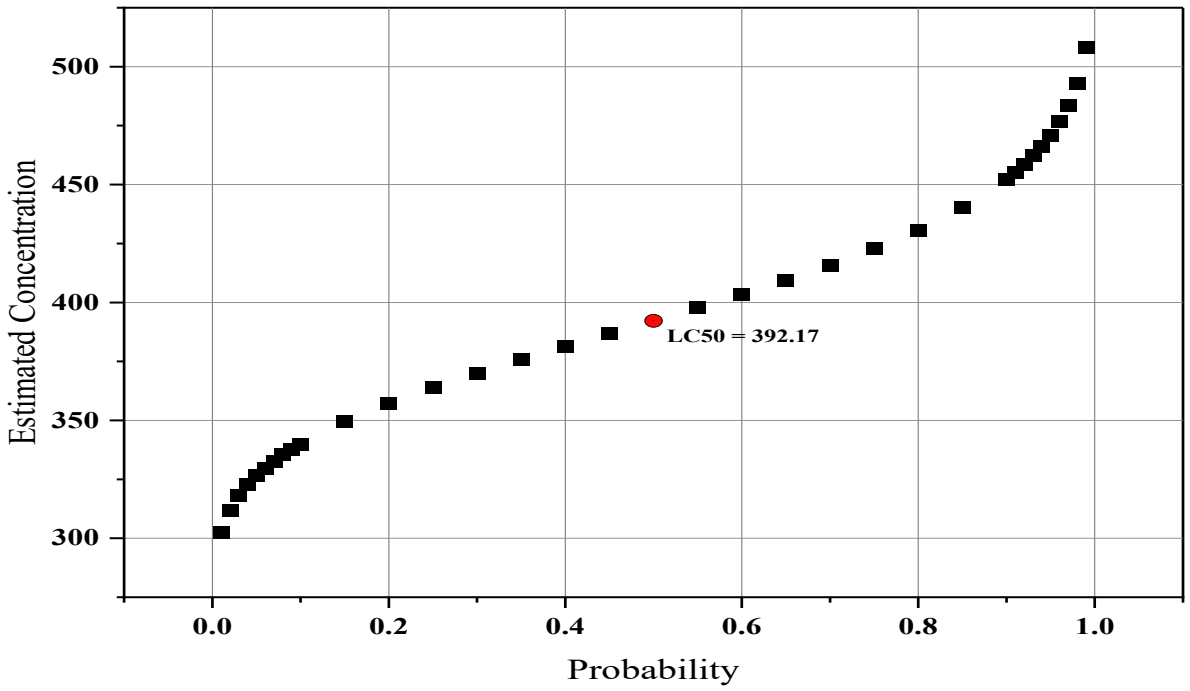

Fig. 1 : Probability of mortality of estimated concentration of 2,4-D which indicates the 96 h LC_{50} as 392.17 mg/l of 2,4 D for *Oreochromis mossambicus*.

2,4 D herbicide resulted in significant changes in almost all haematological indices, especially in the groups exposed to the highest concentrations of 2,4 D herbicide (100 mg/l). The mean control value of the RBC after 30 days of 2,4 D treatment was 2.730 ± 0.031. The experimental group of fishes treated with 2,4 D concentrations of 100 mg/l, 80 mg/l, 60 mg/l, and 40 mg/l showed RBC count as 0.93 ± 0.035, 1.7 ± 0.360, 1.9 ± 0.208, and 2.31 ± 0.277, respectively (P< 0.01) (Table 2).

The mean control value of WBC after 30 days of 2,4 Dtreatment is 700±1.527. The highest WBC content level of 920 ± 2.081 was observed in the fish treated with 100 mg/l of the herbicide. The observed WBC level for 80 mg/l, 60 mg/l, and 40 mg/l is 860±5.56, 790±11.78 and 753±28.1, respectively (P< 0.01) (Table 2).

The mean control value of haemoglobin after 30 days of 2,4 D treatment is 8.2± 0.600. The lowest haemoglobin level of 2.8 ± 0.75 was observed in the fish treated with 100 mg/l of 2,4 D. The observed haemoglobin contents for the concentrations of 40, 60, and 80 mg/l of 2,4-D were 6.1± 0.404, 5.3± 1.026, and 4.6 ±0.655 gm%, respectively (P< 0.01) (Table 2).

PCV level in the control fish is 24.6 ± 0.32 . It was discovered that the amount of PCV in the blood was directly proportional to the amount of exposure. The greatest reduction was observed in the experimental group exposed to 100 mg/l of toxicant followed by groups exposed to 80, 60, and 40 mg/l of the toxicant (Table 2). Among the 40 – 100 mg/l exposure fish groups, the PCV values are19.3 ± 2.179, 16.2 ± 0.208, and 13.9± 0.611 and

Table 2: Haematological parameters in *Oreochromis mossambicus* exposed to 2,4 D for 30 days

Concentration 2,4 D	RBC (million/cu mm)	WBC (cell/cu mm)	Hb (g%)	PCV (g%)	MCV (microns)	MCH	MCHC	Platelet (cell/cu mm)
Control	2.73± 0.031	700± 1.527	8.2± 0.600	24.6± 0.321	90.5± 0.458	30.6± 0.776	33.3± 1.417	18000± 1154.9
40 mg/l	2.31± 0.277	753± 28.1	6.1± 0.404	19.3± 2.179	89.1± 0.568	30.0± 0.868	33.0± 0.838	15000± 13.34
60 mg/l	1.9± 0.208	790± 11.78	5.3± 1.026	16.2± 0.208	84.6± 0.36	30.1± 0.8717	32.9± 0.185	4000± 153.29
80 mg/l	1.7± 0.360	860± 5.56	4.6± 0.655	13.9± 0.611	82.0± 1.153	29.6± 1.101	32.6± 0.793	3000± 59.5
100 mg/l	0.93± 0.035	920± 2.081	2.8± 0.750	8.4± 0.450	81.7± 0.721	27.0± 0.360	32.0± 0.208	2000± 222.8

Values are expressed as mean ± standard error, significant at 1 % level

8.4 ± 0.450, respectively (P < 0.01).

After 30 days of treatment, the control value of MCV is 90.5 ± 0.458. A statistically significant drop in MCV content was detected in fish treated with 100 mg/l, 80 mg/l, 60 mg/l, and 40 mg/l of the toxicant and the values are 81.7±0.721, 82.0 ± 1.153, 84.6 ± 0.36 and 89.1 ± 0.568, respectively (P < 0.01) (Table 2).

The control value of MCH after 30 days of treatment is 30.6 ±0.776. A significant decrease in MCV content (Table 2) has been observed in 100 mg/l, 80 mg/l, 60 mg/l and 40 mg/l treated fish. The values are 27.0±0.360, 29.6±1.01, 30.1±0.8717, 30.0±0.868, respectively at (P < 0.01).

The control value of MCHC after 30 days of treatment is 33.3 ±0.776. A decrease in MCV content has been observed in 100 mg/l, 80 mg/l, 60 mg/l, and 40 mg/l treated fish. The values are 32.0±0.208, 32.6±0.793, 32.9±0.185 and 33.0± 0.838, respectively (Table 2).

The control value of platelet count after 30 days of treatment is 18000 ± 1154.9. A significant decrease in platelet content has been observed in fish treated with 100 mg/l, 80 mg/l, 60 mg/l, 40 mg/l of 2,4 D. The values are 2000 ± 222.8, 3000 ± 59.5, 4000 ± 153.29, 15000 ± 13.34, respectively (P < 0.01) (Table 2).

Effect of 2,4 D on vital organs (*histopathology*):

The gills in the control fish and 40 mg/l 2,4 D exposed fish were normal in appearance. Gill degeneration and congestion were noticed in fish treated with 60 mg/l, 80 mg/l and 100 mg/l 2,4 D (Figs. 2, 3).

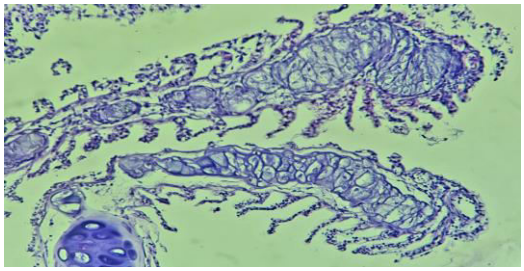

Fig. 2: Degeneration of gill in 60 mg/l 2,4 D exposed fish. Hematoxylin-Eosin X 400.

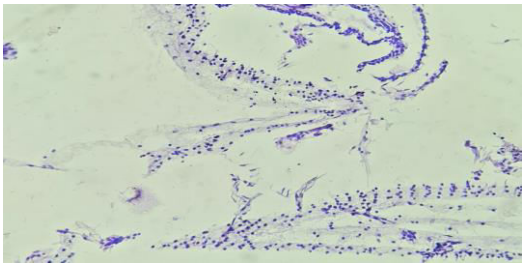

Fig. 3: Degeneration of gill in 100 mg/l 2,4 D exposed fish. Hematoxylin-Eosin X 400.

In the control and 40 mg/l 2,4 D treated fish, the liver parenchyma appeared normal. After 60 mg/l 2,4 D treatment, the liver parenchyma showed congestion and fatty degeneration (Fig. 4). However, hepatic parenchyma with features of severe fatty degeneration and congestion was noticed in the fish treated with 80 mg/l and 100 mg/l of 2,4-D (Fig. 5).

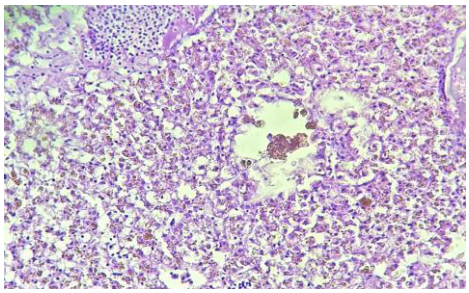

Fig. 4: Liver of 60 mg/l 2,4 D exposed fish showing congestion and fatty degeneration. Hematoxylin-Eosin X 100.

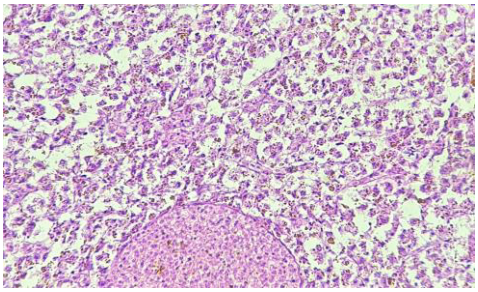

Fig. 5: Liver of 100 mg/l 2,4 D exposed fish showing severe fatty degeneration. Hematoxylin-Eosin X 100.

Discussion

In the present study a decrease in RBC has been noticed in fish treated with 2,4 D. Wahbi *et al*. (2004) and Zaki *et al.* (2008) attributed the decrease in the RBC to heamolytic crisis that results in severe anemia in fish exposed to heavy metals and herbicide. Furthermore, the reduction of RBC also leads to development of hypoxic condition which in turn leads to increase in destruction of RBC or decrease in rate of formation of RBC due to non-availability of Hb content in cellular medium (Chen *et al.,* 2004). According to Kumar *et al.* (2004) White blood cells play a major role in the defense mechanism of the fish. Fink and Salibian (2005) reported that WBC increase could be due to an induced proliferation as a result of the chemical toxicity of pluripotential hematopoietic cells that in turn may be a consequence of a depletion of circulating differentiated cells. The increase in WBC count in this study indicates the stress condition of the fish caused by 2,4 D which might have produced hypoxia and gill damage as suggested by Ramesh *et al.* (2009).

2,4 D exposure caused a decrease in Hb content of the fish. A considerable fall in haemoglobin concentration can be due to compensatory response that limits the oxygen-carrying capacity in order to sustain gas exchange in damaged gill lamella (Soni *et al*., 2018). Hematological changes in *Clarias batrachus* were attributed to rapid destruction of red blood cells by the toxicant 2,4-D exposure. A reduction in the Hb% could lead to a decrease in blood oxygen-carrying capacity. Prolonged reduction in haemoglobin content is deleterious to oxygen transport, and blood dyscrasia and degeneration of the erythrocytes could be ascribed as a pathological condition in fishes exposed to toxicants. Our results are in good concurrence with the earlier works of Buckley *et al.* (1976) and Palanisamy *et al*. (2011). The PCV values decrease when a fish loses appetite or becomes diseased or stressed (Annune and Ahuma, 1998). In the present study, the decrease in the level of Haemoglobin and PCV after exposure to 2,4 D suggests a haemodilution mechanism possibly due to gill damage or impaired osmoregulation. The haemodilution has been interpreted as a mechanism that reduces the concentration of the irritating factor in the circulatory system (Smith *et al*., 1979; Bhagwant, 1999). The present findings are in agreement with observations of Akinrotimi *et al.* (2009). The decrease in the PCV indicates the worsening of the condition of the organism and development of anaemia.

In the present study there is a decrease in MCV of fish after 2,4 D treatment. According to Soni *et al.* (2018), the MCV of the experimental fish (*Clarias batrachus*) was significantly reduced compared to the control fish subjected to 2, 4 D

herbicide. According to Safahieh *et al.* (2012), the MCV count was lowered in *Mesopotamichthys sharpeyi* exposed to paraquat herbicide compared to the control, which they attributed to low oxygen consumption in the fish. Various researchers reported that the cells released from the spleen, the erythropoietic organ, might have lowered the MCV value (Ololade and Oginni, 2010; Singh *et al*., 2010). According to Gunashekaran and Vellaichamy (2019), the decrease in MCV is due to the defence against the toxicant. Sivanandan and Binukumari (2021) have reported that after Malathion exposure hematological indices including MCV and MCH, depicted a considerable decrease ($P<0.05$). The alterations in MCV and MCH were ascribed to hemolysis and impairment in haemoglobin synthesis (Marei *et al.,* 1998; Shah, 2006). The MCH is a good indicator of red blood cell swelling (Wepener, 1992, Bhagwant and Bhikajee, 2009). According to Atmanalp *et al.* (2002), decreased MCHC indicates hypochronic microlytic anemia. According to Latha and Ramachandra (2018), a decrease in MCHC indicates that the haemoglobin concentration in RBC is reduced. The decrease in the MCHC values in the present study may be due to swelling of RBC or a decrease in haemoglobin synthesis. The present results are in line with the findings of Siakpere (2011).

Platelets responsible for blood clotting in fish; a slight decrease in values observed in this study may signify the effect on platelet (thrombocyte) production (Soni *et al.*, 2018).

Fish gills are in close contact with the external environment and perform functions such as respiration and osmoregulation. As a result, any change in water quality has a negative impact on gill function (Fernandes and Mazon 2003). Vigário and Sabóia-Morais (2013) conducted histochemical analysis of fish exposed to 40 µg/l 2,4-D and reported hypertrophic mucous cells along gill filaments and congestion. Congestion and degeneration have been noticed in the gills of *Oreochromis mossambicus* after exposure to 2,4 D. Similar changes have been observed by Maurya and Malik (2016) and Winkler *et al.,* (2001). Since gills are the respiratory and osmoregulatory organ of fish, the histopathological changes of the gills might impair the respiratory function of the gills and degeneration of gills reduce respiratory surface area resulting in hypoxia and respiratory failure problems (Alazemi *et al.*, 1996; Yasser and Naser 2011) which badly affects the physiology and may lead to the death of fish (Mohamed, 2003).

Hinton and Laurén (1990) reported that liver of fish exposed to 10 and 20 µl/l of 2,4 D herbicide showed hepatic tissue alteration in the core, and central region and vacuolated hepatocytes were observed with a spongy aspect are more often affected by toxic substances than those from other regions. According to Ortiz *et al*. (2003), after exposure to toxic substances, hepatocytes showed vacuoles that presented as clear vesicles occupying the entire cytoplasm, thus indicating the toxic potential of 2,4 D herbicide. Soni *et al.* (2019) reported extensive fatty degeneration of hepatocytes showing shrinkage of hepatocytes, accumulation of fat the histopathological changes in liver of the fish *Clarias batrachus* exposed to 2,4 D (34.64 µl/l). Pesticide-induced morphologic and histopathological changes in the fish liver have been studied which revealed that toxic compounds cause severe damage to the liver cells (Ortiz *et al*., 2002). Hepatocytes are the most abundant cell types in the liver and perform the majority of the liver's essential functions, such as converting glucose to glycogen, regulating lipids, and deamination of amino acids. Herbicide exposure will result in damage to these functions (Wright *et al*., 2004).

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