International Journal of Research (IJR)
e-ISSN: 2348-6848, p- ISSN: 2348-795X Volume 2, Issue 4, April 2015
Available at http://internationaljournalofresearch.org

Acute exposure to Diisononyl phthalate (DINP) influenced histopathological and behavioural modification on the freshwater fish, *Oreochromis mossambicus* (Peters, 1852)

V. Revathy and K.C. Chitra*
Department of Zoology, University of Calicut, Malappuram District, Kerala, 673 635, India
* Corresponding author
e-mail: kcchitra@yahoo.com
Phone: +91-9495135330

ABSTRACT

The present study reports on the acute toxic effect of diisononyl phthalate (DINP) in the freshwater fish, Oreochromis mossambicus. Fishes were exposed to DINP at six different concentrations – 50 ppm, 100 ppm, 150 ppm, 200 ppm, 250 ppm, 300 ppm/ L dissolved in propylene glycol as solvent maintained for 96 h along with positive and negative control groups. Behavioural changes in the animals were continuously monitored in all experimental groups throughout the study. At the end of every treatment the weights of the animals were recorded along with the gill and liver tissue weights. The tissues that were immediately fixed in buffered formalin for histopathological alterations was observed through Trinocular research microscope and photomicrographed.

Key words:
DINP; gill; liver; histopathology; behaviour; *Oreochromis*

1. INTRODUCTION

Phthalates or phthalate esters has been released from soft polyvinyl chloride (PVC) plastics by surface contact, especially where mechanical pressure is applied like chewing of PVC teether, as they are not tightly bound to the plastic, but are present as mobile components of the plastic matrix. Phthalates, which make up 10–40% of the total weight of a toy, have been banned in several countries as they cause serious potential health effects (Duty et al., 2003). But in India, the uses of phthalates in children's toys that are available in the local market have been noticed where the soft toys had high level of phthalates than the hard toys (Johnson et al., 2011).

Diisononyl phthalate (DINP), one of the high molecular weight phthalates is widely used as a general purpose plasticizer in order to soften the PVC plastics. DINP, an oily viscous liquid belongs to the class of dialkyl phthalate esters that represents a complex of branched, predominantly C-9 isomers. It has an extensive range of applications in indoor items as wires and cables, floor etc as well as in outdoor products as roofing material, coated fabric, hoses, car under-coating, shoe soles, sealing, paints and lacquers etc. and has also been found in toys and child care articles as teethers, rattles and bottle nipples (ECB, 2003).

As humans are highly exposed to such phthalates consequently results in severe health impacts. Recently there has been an increasing concern regarding the toxic effects of phthalates and there is an insufficient data stating their toxic impacts on aquatic ecosystem especially to fish community. It was also well known that the study of animal behaviour can be considered as one of the early

International Journal of Research (IJR)
e-ISSN: 2348-6848, p- ISSN: 2348-795X Volume 2, Issue 4, April 2015
Available at http://internationaljournalofresearch.org

warning bio-indicators to certain pollutants. Moreover, the histopathological observations are widely measured as a noteworthy and promising parameter to understand the extent to which the changes in the structural organisations are occurring in the organs due to the exposure of such pollutants. Therefore, the present study was aimed to evaluate the acute toxicity effect of DINP in the freshwater fish, *Oreochromis mossambicus* at several different concentrations.

2. MATERIALS AND METHODS

Freshwater fish, *Oreochromis mossambicus* weighing 3.5 ± 0.75 g and length 5.5 ± 1.5 cm were collected from a fish farm, Safa Aquarium, Kozhikode, Kerala. Fishes were transported to the laboratory with least disturbance and were acclimatized to the laboratory conditions prior to experiments with constant supply of water and good lighting system in well-aerated tubs (40 L capacity), which was dechlorinated and sustained with fresh water flow and waste water discharge.

The physico-chemical features of the tap water were estimated as per APHA (1998). Water temperature in the test ranged from 28 ± 2°C during the experiment, oxygen saturation of water ranged between 70 and 100 %, pH is 7.6 which were monitored using a standardized measures.

Diisononyl phthalate (1,2, - Benzenedicarboxylic acid or DINP; CAS No. 28553120) of 99% purity was obtained from Sigma Aldrich chemical Co., USA. Propylene glycol was used as a solvent to dissolve DINP where 16 µl of 1 M propylene glycol was sufficient to dissolve 300 ppm DINP. Toxicant (DINP) was dissolved in propylene glycol by sonication at 50 Hz for 5 minutes with 30sec pulse interval. Experiments were carried out with 10 animals per group maintaining 3 groups of animals. In Group I the same concentration of propylene glycol per litre that was used to dissolve DINP (16 µl; 1 M) was treated to fishes and it was maintained for 96 h as positive control. In Group II, ten animals were retained in toxicant/ solvent-free water for 96 h. Then Group III was maintained with six subgroups at different concentrations of DINP (50 ppm, 100 ppm, 150 ppm, 200 ppm, 250 ppm, 300 ppm/ L dissolved in propylene glycol; 10 animals per subgroups) for 96 h. The fishes were not fed a day prior to and during the experiments in order to reduce fecal and excess food contaminating the test solution. All experimental tubs were properly aerated using tubed motorized pumps. Monofilament netting was used to cover the tanks to prevent the specimens from jumping out of test solutions. The mortality as well as the behaviour of fishes was recorded throughout the study.

3. RESULTS AND DISCUSSION

Exposure to DINP for 96 h did not caused treatment related alterations in the body weights and liver or gill weights as compared with the control groups. Similarly fishes treated with propylene glycol alone did not caused any change in the body weights or organ weights for 96 h when compared to the negative control group (Table 1). Thus it was clear that the selected concentrations varying from 50 to 300 ppm/ L did not induced treatment related lethality in fishes. Similarly no mortality was observed in all treatment groups throughout the study. In one of the studies it was reported that among the mixture of six phthalates as DBP, BBP, DEHP, DIDP, DINP and DNOP it was found that DBP was more toxic followed by BBP and the LC50 reported was 0.63 ppm/ L and 0.72 ppm/ L respectively in zebra fish, *Danio rerio* (Chen et al., 2014). The highest soluble concentration of DINP is 300 ppm/ L, therefore, in the present study 300 ppm/ L was taken as highest dose and thus median lethal

concentration (LC_{50}) could not be found as no mortality was noticed with the selected concentrations varying from 50 to 300 ppm/ L for 96 h.

Propylene glycol is a viscous, colorless, organic solvent used primarily in the production of polymers, pharmaceuticals, veterinary medicines, approved food additive for animal feed and also used in various edible items during food processing. Unlike the other organic solvents, propylene glycol in non-toxic, readily miscible in water and do not cause stress to the animals and the Food and Drug Administration generally classified and recognized as safe and acceptable for use in flavorings, drugs, and cosmetics, and as a direct food additive. According to the World Health Organization, the acceptable dietary intake of propylene glycol is 25 mg of propylene glycol per kilogram body weight in humans (ATSDR, 1997). Therefore, in the present study propylene glycol was used as a vehicle to dissolve DINP and found no effects on the body weight, organ weights, behaviour or histological parameters in fish when treated for 96 h.

Behaviour is considered as one of the open frame bio-indicators that are ultimate for assessing the effects of aquatic pollutants on fish population. Changes in the normal behaviour are an adaptive mechanism which allows the fish to maintain normal homeostatic state and also to escape or adjust from the stress condition (Gormley and Teather, 2003). In the present study it was noticed that immediately after the exposure to DINP the fishes at all concentrations showed an aggressive behaviour like fighting each other, hitting on the walls of the tub, restlessness followed by loss of balance and such activities remained per se for some time. During the later stage of the treatment fishes showed anorexia, unresponsive behaviour and darkening of skin. Introduction to toxicant in the aquatic environment could be the cause of hyperactivity of the animal at all concentrations of DINP and it was considered as a primary and principal sign of failure of nervous system and there is also a possibility of changes in physiological and biochemical activities (Matsumura, 1975). The interruption of the functioning of nervous system of fish might be the cause of slow and lethargic swimming, erratic swimming and loss of equilibrium in the later hours of DINP exposure. Thus the release of high molecular weight phthalate plasticizers could cause ecological imbalance in aquatic environment where inappropriate behavioural responses was served as a bio-indicator to prove that DINP seriously affect the health status of the fish, an excellent model of aquatic ecosystem. Animals when exposed to propylene glycol did not show any abnormal behaviour as noticed in DINP-treated groups.

Histopathological parameters are considered as the most reliable biomarkers to monitor stress in fish and also to evaluate health status of fish exposed to contaminants, both in the laboratory and field studies. One of the great advantages of using histopathological biomarkers in environmental monitoring is it allows examining specific target organs, including gills, liver etc, that are responsible for vital functions, such as respiration, accumulation and biotransformation of toxicants in the fish. Recently researchers are more concerted on the study of aquatic toxicants frequently been tested on gills. General structure of the gills consisted of primary and secondary gill lamellae (Fig 1a) and the treatment with propylene glycol as vehicle did not alter the general organization of gills (Fig 2a). The secondary lamellae were lined by squamous epithelial cells. Between secondary lamellae, the primary lamellae are lined by stratified epithelium and mucous cells,

while the chloride cells were scattered in the base of lamellae and in the interlamellar region. In treatment groups at 50 ppm DINP/ L the lesions were observed in the gills included lamellar swelling, complete destruction of gill lamellae, and blubbing in gill filaments were observed (Fig 3a). Uplifting of gill epithelium and interlamellar space was observed at 100 ppm DINP/ L (Fig 4a). In 150 ppm/ L DINP-treated groups showed a prominent congestion in primary and secondary gill lamellae (Fig 5a). Moreover, hyperplasia of the epithelial cells of gill filaments, decrease of interlamellar space, hypertrophy and hyperplasia of pavement and chloride cells was noticed at 200 ppm DINP/ L (Fig 6a). Lifting and hyperplasia of lamellar epithelium could be the defensive mechanism of the fish against the aquatic toxicants and may serve as a barrier to the entrance of toxicant (Pandey et al., 2008). Complete destruction of gill arches and blubbing in gill filaments were observed at 250 ppm/ L and 300 ppm/ L concentrations (Fig 7a and 8a). The most common cause of cellular degeneration in gill filaments is oxygen deficiency as a result of gill toxicity (Mohamed, 2009). Gills are considered as the primary target organ of aquatic pollutants due to the constant contact with the external milieu (Perry and Laurent, 1993).

Liver is the most important organ associated with the detoxification and biotransformation process and thus it is also one of the organs most affected by aquatic contaminants (Rodrigues and Fanta, 1998).Thus evaluation of histological changes in fish liver is a highly sensitive and accurate way to assess the effects of aquatic pollutants in field and laboratory studies. In the present study the liver of control fish and those treated with propylene glycol revealed normal polygonal paranchymatous cells with cytoplasm and a central spherical nucleus (Fig 1b and 2b). In DINP at 50 and 100 ppm/ L concentrations the hepatic parenchyma is not arranged into distinct lobules and they appeared as segmented fragments (Fig 3b and 4b) whereas at 150 ppm/ L concentration showed erythrocyte infiltration into blood sinusoids (Fig 5b). DINP at 200 ppm/ L concentration showed necrosis (Fig 6b) and at 250 and 300 ppm/ L concentrations showed intravascular hemolysis, nuclear pyknosis and irregular elongated nucleus (Fig 7b and 8b). All the above changes in fish hepatocytes after DINP treatment reflects that liver fails to detoxify and degrade DINP even at lowest concentration and could subsequently resulted in structural damage.

4. CONCLUSION

The present findings suggest that DINP even at least concentration and with the solubility up to 300 ppm/ L when exposed to aquatic environment could cause hazardous effect to aquatic animals including fish even though it is not causing any lethal effect as mortality. But these concentrations are identified to highly affect the behaviour as well as it vastly influences the alterations in normal architecture of vital organs as gill and liver. Therefore, even though median lethal concentration (LC_{50}) was not found in these concentrations, DINP in the aquatic environment still at less concentration are not safe and may pose threaten to aquatic fauna thus its usage or exposure could be made limited.

ACKNOWLEDGEMENT

The authors acknowledge UGC-SAP/ BSR for utilizing the equipments during this study.

5. REFERENCES

[1.] Agency for Toxic Substances and Disease Registry (ATSDR). (1997). Public health statement for propylene glycol. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

[2.] APHA. (1998). Standard methods for the examination of water and waste water, 20th Edition, Washington, DC.

[3.] Chen X, Xu S, Tan T, Lee ST, Cheng SH,Lee FWF, Xu SJL, Ho KC. (2014). Toxicity and estrogenic endocrine disrupting activity of phthalates and their mixtures. *Int. J. Environ. Res. Public Health.* 11: 3156-3168.

[4.] Duty SM, Silva MJ, Barr DB, Brock JW, Ryan L, Chen Z, Herrick RF, Christiani DC, Hauser R. (2003). Phthalate exposure and human semen parameters. *Epidemiology* 14: 269–277.

[5.] European Chemicals Bureau (ECB). (2003). European Union Risk Assessment Report. 1,2-benzenedicarboxylic acid, di-C8-10-branched alkyl esters, C9 rich and di-"isononyl" phthalate (DINP). Volume 35. Institute for Health and Consumer Protection, European Chemicals Bureau, European Commission.

[6.] Gormley KL, Teather KL. (2003). Developmental, behavioral, and reproductive effects experienced by Japanese medaka (*Oryzias latipes*) in response to short-term exposure to endosulfan. *Ecotoxicol. Environ. Saf.* 54: 330-338.

[7.] Johnson S, Saikia N, Sahu R. (2011). Phthalates in Toys Available in Indian Market. *Bull. Environ. Contam. Toxicol.* 86: 621–626.

[8.] Matsumura F. (1975). Toxicology of Insecticides. 2nd Edn. Plenum Press NY. pp 503.

[9.] Mohamed FAS. (2009). Histopathological studies on Tilapia zillii and Solea vulgaris from Lake Qarun, Egypt. *World J. Fish Mar. Sci.* 1: 29-39.

[10.] Pandey S, Ansari PS, Ali RA, Kaur M, Hayat M, Ahmad F, Raisuddin S. (2008). Effects of exposure to multiple trace metals on biochemical, histological and ultrastructural features of gills of a freshwater fish, *Channa punctata* Bloch. *J. Chemico-Biological Interactions* 174: 183–192.

[11.] Perry SF, Laurent P. (1993). Environmental effects on fish gill structure and function. In: Rankin, J.C., Jensen, F.B. (Eds.), *Fish Ecophysiology*. London, pp. 231–264.

[12.] 12. Rodrigues EL, Fanta E. (1998). Liver histopathology of the fish *Brachydanio rerio* after acute exposure to sublethal levels of the organophosphate Dimetoato 500. *Revista Brasileira de Zoologia,* 15, 441-450.

International Journal of Research
International Journal of Research (IJR)
e-ISSN: 2348-6848, p- ISSN: 2348-795X Volume 2, Issue 4, April 2015
Available at http://internationaljournalofresearch.org

Table 1 Effect of diisononyl phthalate (DINP) on the body weights and organ weights of freshwater fish, *Oreochromis mossambicus*

DINP (ppm/ L)	Body weight (g)	Weight of gill (mg)	Weight of liver (mg)
Control	3.40 ± 0.75	120 ± 0.22	50 ± 0.58
Propylene glycol (1M; 16µL)	3.25 ± 0.80	123 ± 0.24	51 ± 0.64
DINP (50 ppm/L)	3.27 ± 0.64	124 ± 0.30	51 ± 0.55
DINP (100 ppm/L)	3.52 ± 0.55	124 ± 0.28	51 ± 0.63
DINP (150 ppm/L)	3.61 ± 0.46	125 ± 0.25	52 ± 0.76
DINP (200 ppm/L)	3.47 ± 0.88	126 ± 0.23	54 ± 0.55
DINP (250 ppm/L)	3.62 ± 0.65	124 ± 0.32	52 ± 0.72
DINP (300 ppm/L)	3.55 ± 0.71	123 ± 0.28	53 ± 0.83

Data are expressed as mean ± SD for ten-animals/ group

Fig 1a Photomicrograph (10X magnification) of control fish showing the normal architecture of gill

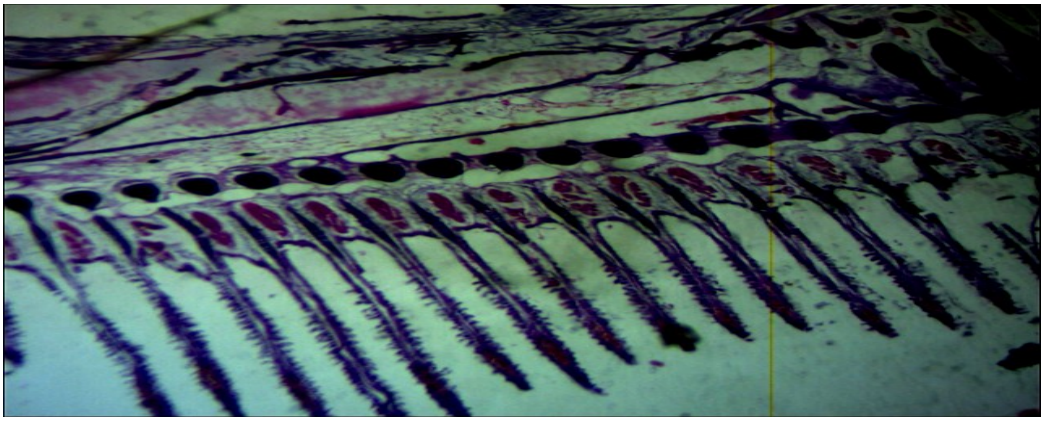

Fig 1b Photomicrograph (40X magnification) of control fish showing the normal architecture of liver

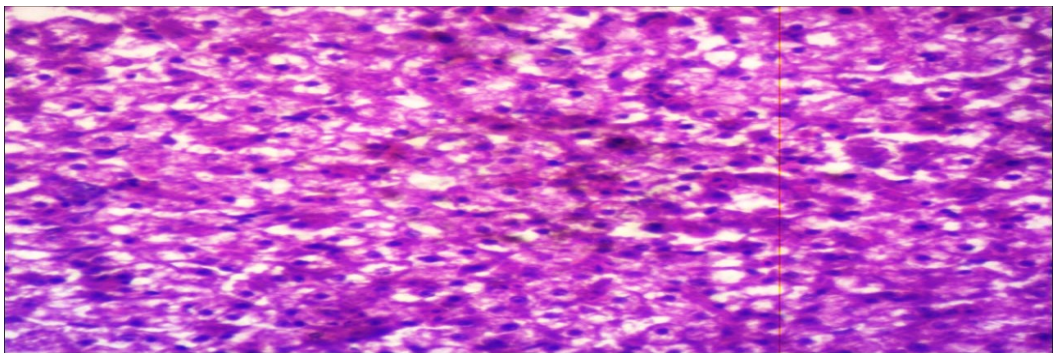

Fig 2a Photomicrograph (10X magnification) showing the normal structure of gill when treated with Propylene glycol

International Journal of Research
International Journal of Research (IJR)
e-ISSN: 2348-6848, p- ISSN: 2348-795X Volume 2, Issue 4, April 2015
Available at http://internationaljournalofresearch.org

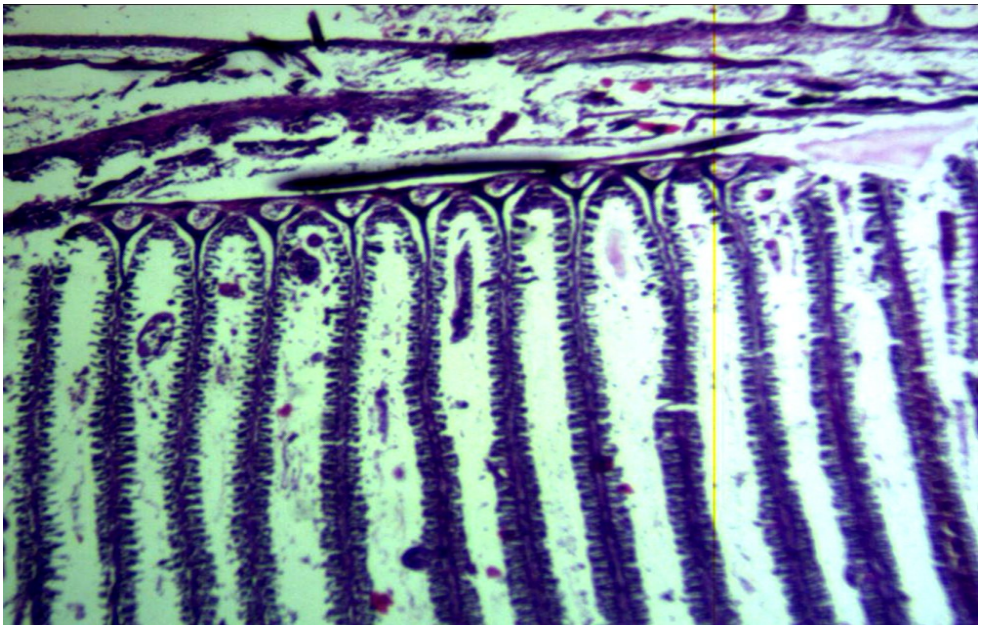

Fig 2b Photomicrograph (40X magnification) showing the normal structure of liver when treated with Propylene glycol

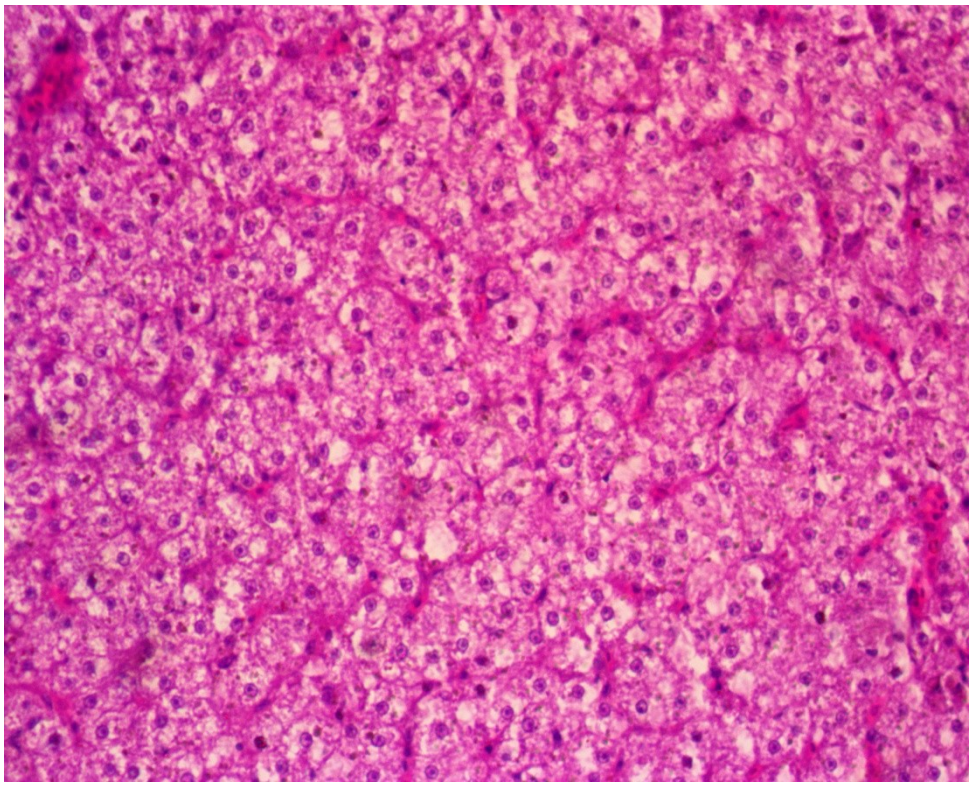

Fig 3a Photomicrograph (40X magnification) showing lamellar swelling, destruction of gill lamellae and blubbing in gill filaments of fish exposed to DINP at 50 ppm/ L concentration for 96 h

International Journal of Research
International Journal of Research (IJR)
e-ISSN: 2348-6848, p- ISSN: 2348-795X Volume 2, Issue 4, April 2015
Available at http://internationaljournalofresearch.org

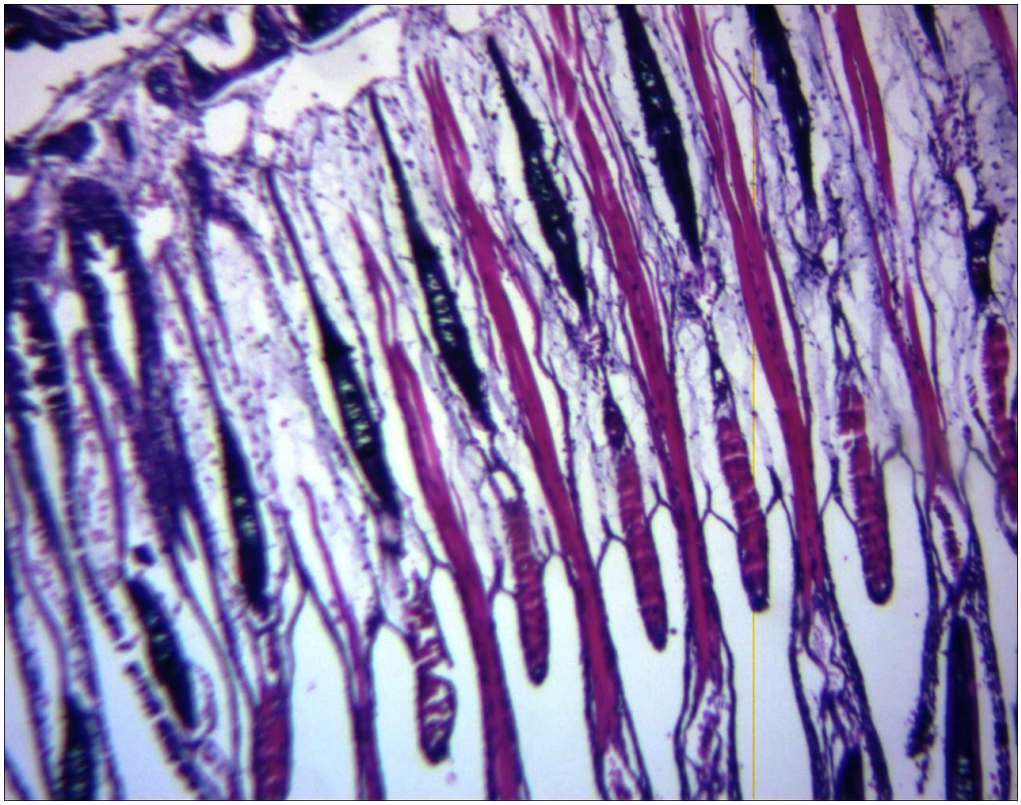

Fig 3b Photomicrograph (40X magnification) showing segmented hepatic parenchyma of fish exposed to DINP at 50 ppm/ L concentration for 96 h

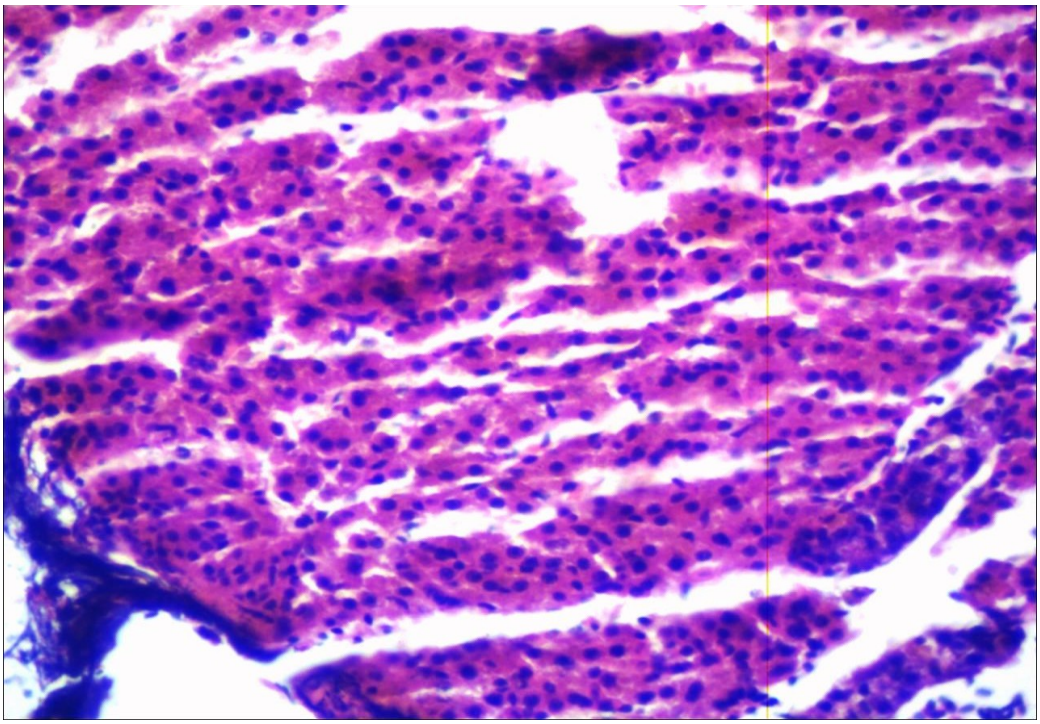

Fig 4a Photomicrograph (10X magnification) showing uplifting of gill epithelium and interlamellar space in fish exposed to DINP at 100 ppm/ L concentration for 96 h

International Journal of Research
International Journal of Research (IJR)
e-ISSN: 2348-6848, p- ISSN: 2348-795X Volume 2, Issue 4, April 2015
Available at http://internationaljournalofresearch.org

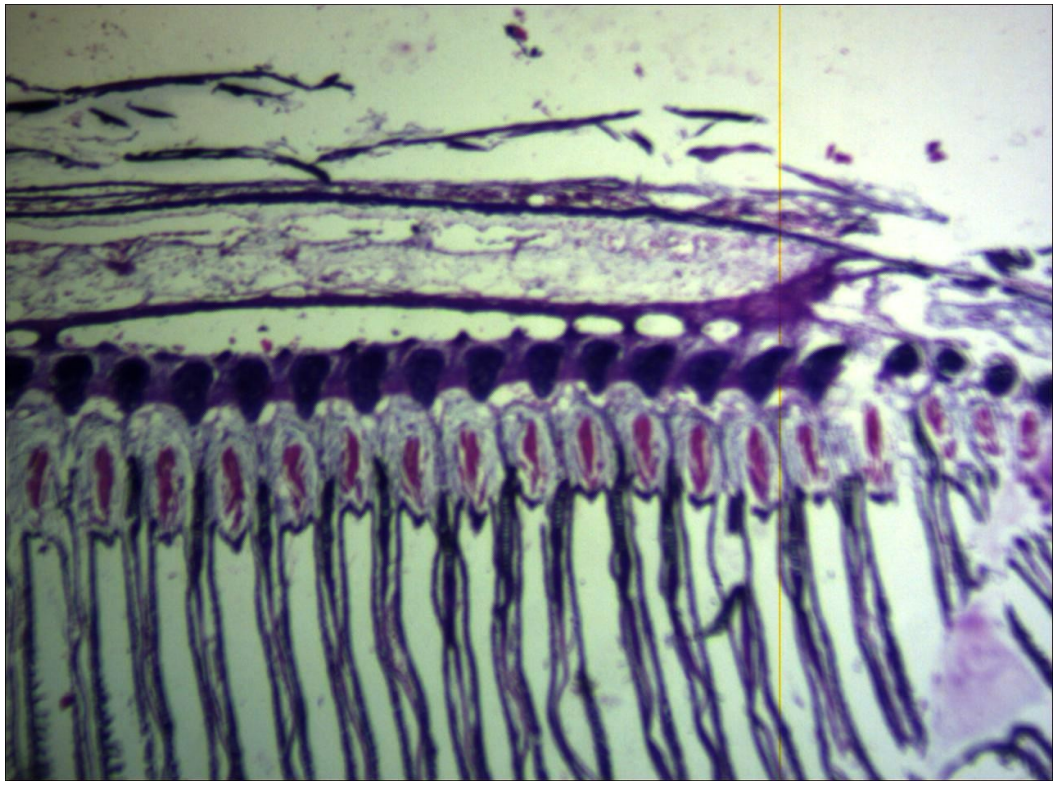

Fig 4b Photomicrograph (40X magnification) showing segmented hepatic parenchyma of fish exposed to DINP at 100 ppm/ L concentration for 96 h

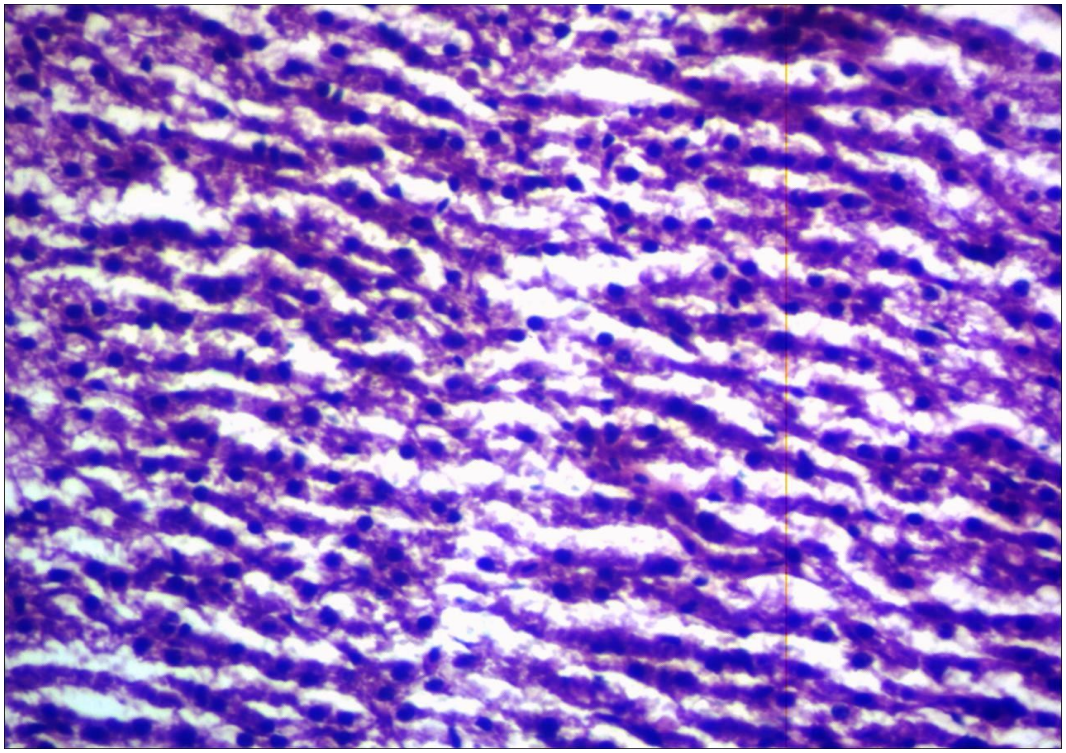

Fig 5a Photomicrograph (40X magnification) showing congestion of primary and secondary gill lamellae of fish exposed to DINP at 150 ppm/ L concentration for 96 h

International Journal of Research
International Journal of Research (IJR)
e-ISSN: 2348-6848, p- ISSN: 2348-795X Volume 2, Issue 4, April 2015
Available at http://internationaljournalofresearch.org

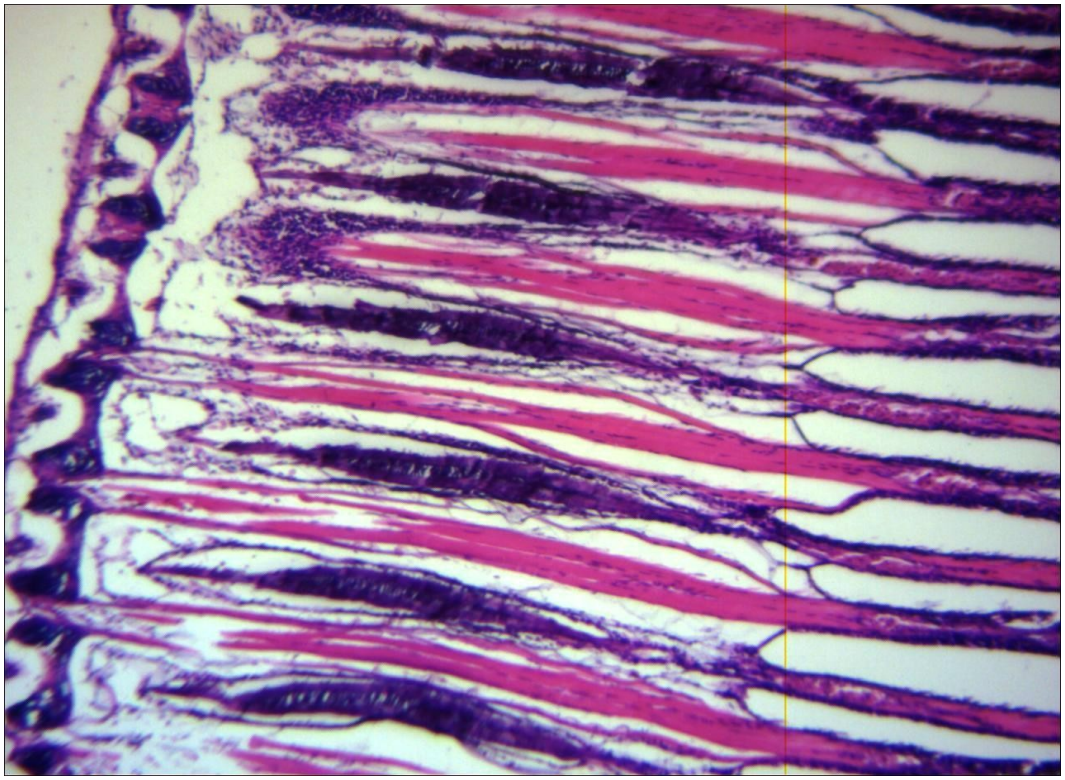

Fig 5b Photomicrograph (40X magnification) showing erythrocyte infiltration into blood sinusoids in fish exposed to DINP at 150 ppm/ L concentration for 96 h

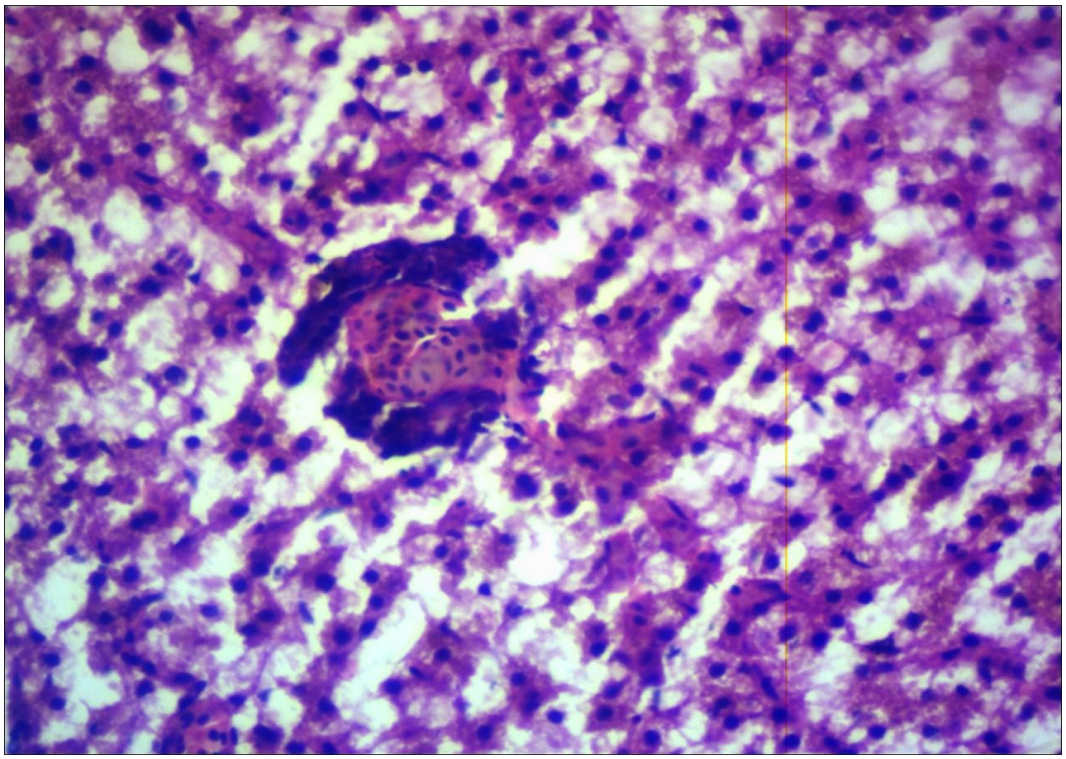

Fig 6a Photomicrograph (40X magnification) showing hypertrophy and hyperplasia of gill lamellae in fish exposed to DINP at 200 ppm/ L concentration for 96 h

International Journal of Research
International Journal of Research (IJR)
e-ISSN: 2348-6848, p- ISSN: 2348-795X Volume 2, Issue 4, April 2015
Available at http://internationaljournalofresearch.org

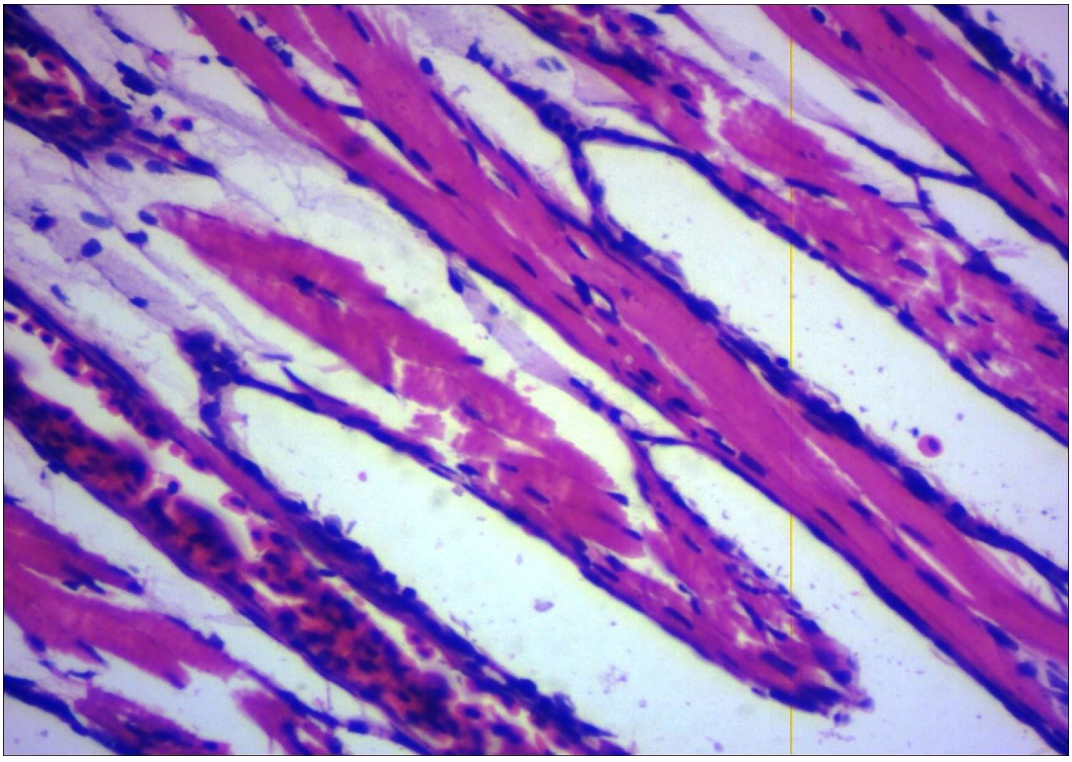

Fig 6b Photomicrograph (40X magnification) showing hepatic necrosis in fish exposed to DINP at 200 ppm/ L concentration for 96 h

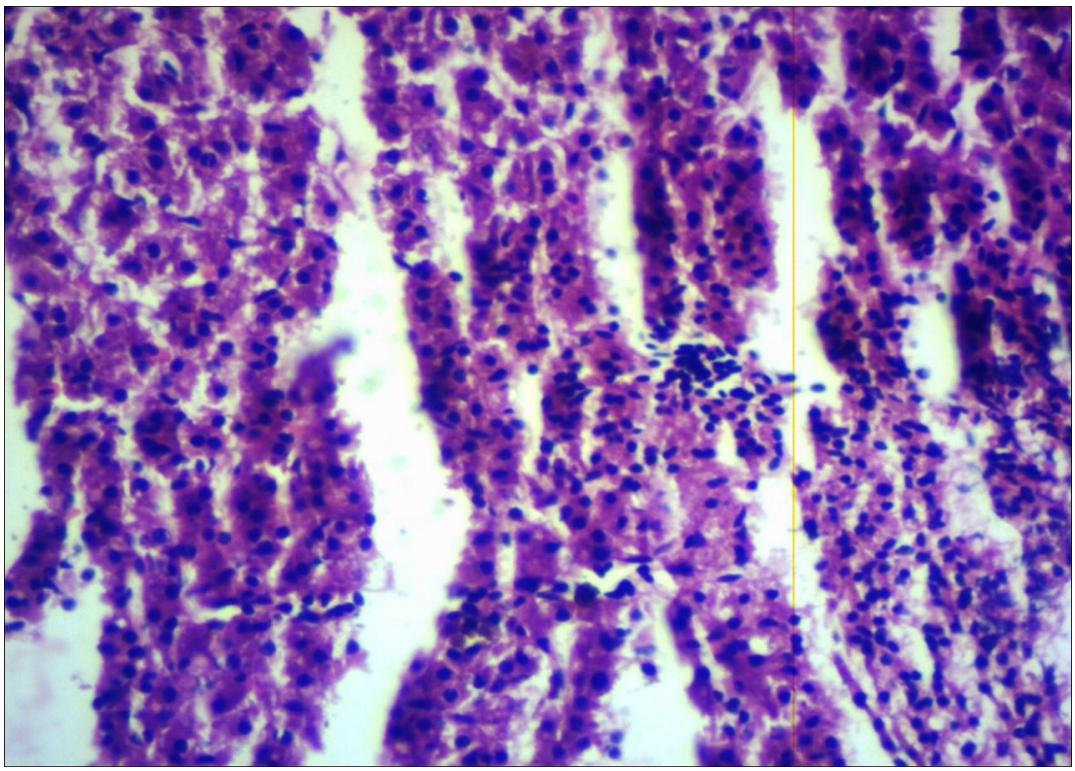

Fig 7a Photomicrograph (40X magnification) showing destruction of gill arches in fish exposed to DINP at 250 ppm/ L concentration for 96 h

International Journal of Research
International Journal of Research (IJR)
e-ISSN: 2348-6848, p- ISSN: 2348-795X Volume 2, Issue 4, April 2015
Available at http://internationaljournalofresearch.org

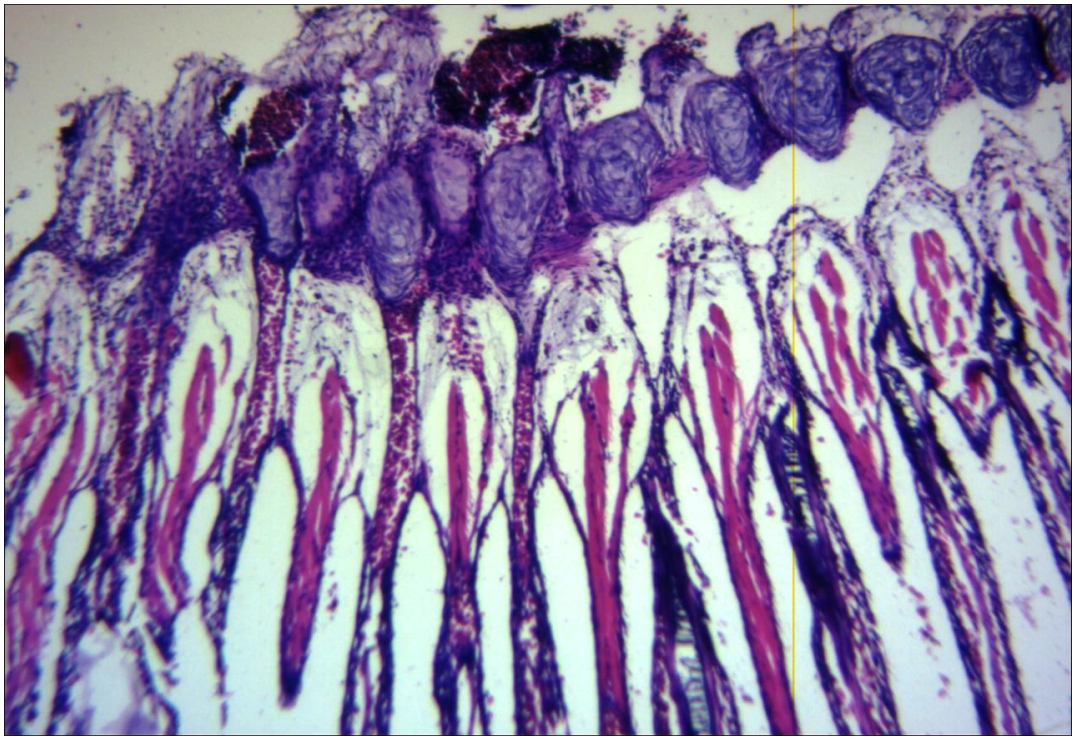

Fig 7b Photomicrograph (40X magnification) showing intravascular hemolysis, nuclear pyknosis and irregular elongated nucleus in fish liver exposed to DINP at 250 ppm/ L concentration for 96 h

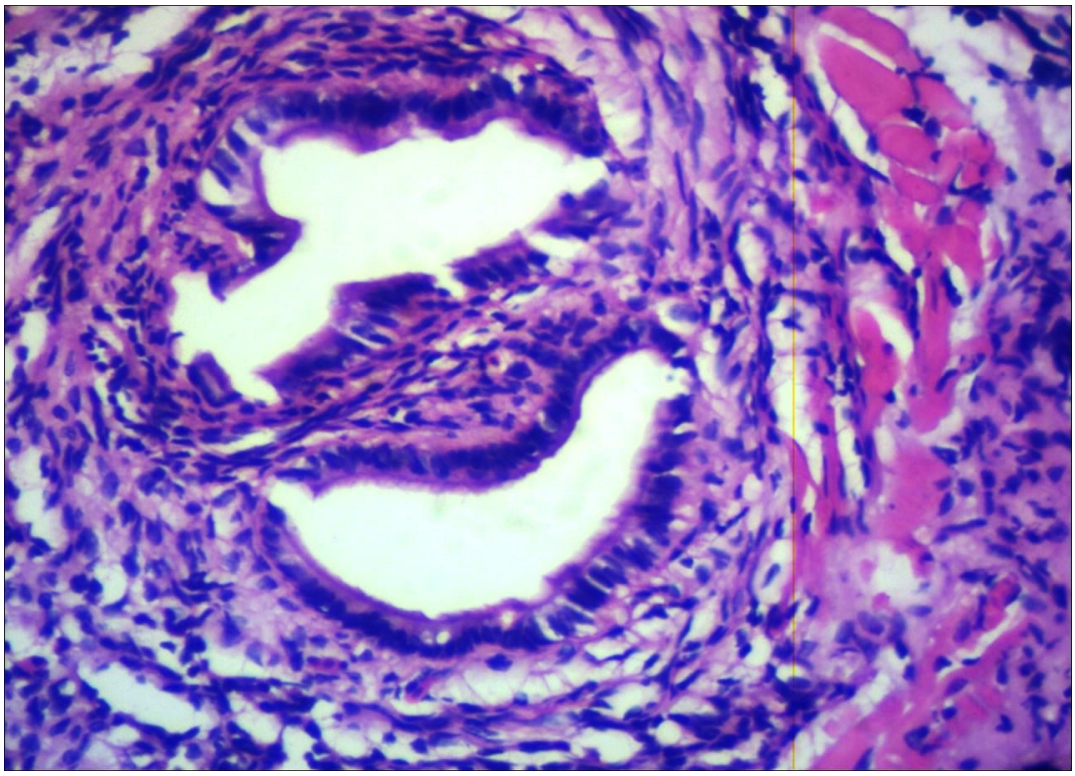

Fig 8a Photomicrograph (40X magnification) showing destruction of gill arches in fish exposed to DINP at 300 ppm/ L concentration for 96 h

International Journal of Research
International Journal of Research (IJR)
e-ISSN: 2348-6848, p- ISSN: 2348-795X Volume 2, Issue 4, April 2015
Available at http://internationaljournalofresearch.org

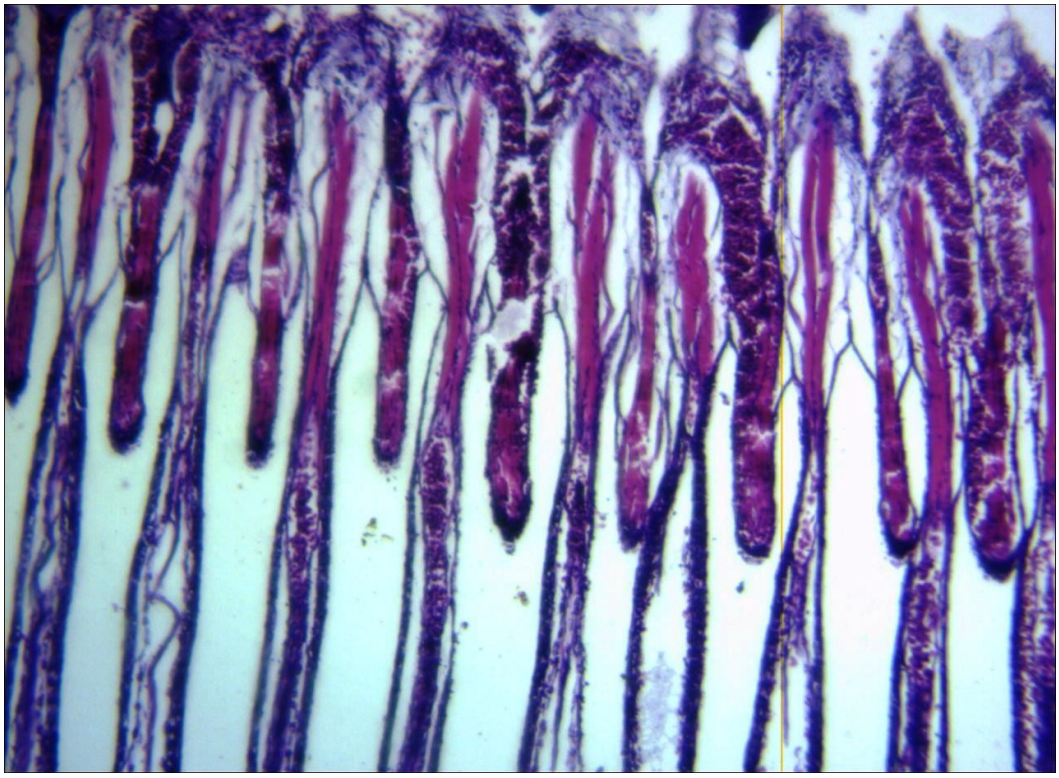

Fig 8b Photomicrograph (40X magnification) showing intravascular hemolysis, nuclear pyknosis and irregular elongated nucleus in fish liver exposed to DINP at 300 ppm/ L concentration for 96 h