Assessment of Acute Toxicity and Histopathological Impact Of Di-n-Butyl Phthalate (DnBP) On Fresh Water Cyprinid Fish Crucian Carp (*Carassius Carassius* L.)

[1]Anjum Afshan, [2] Md Niamat Ali, [3]Farooz Ahmad Bhat
[1]Research Scholar ,Cytogenetics and Molecular Biology Research Laboratory, Centre of Research for Development (CORD), University of Kashmir, Srinagar-190006, J & K, India.
[2] Associate Professor, Cytogenetics and Molecular Biology Research Laboratory, Centre of Research for Development (CORD), University of Kashmir, Srinagar-190006, J & K, India.
[3] Associate Professor, Division of Fisheries Research Management, Faculty of Fisheries, Sher-e-Kashmir University of Agricultural Science and Technology- Kashmir-190025, J & K, India.

Abstract: Di-n-butyl phthalate (DnBP) is a manufactured chemical, commonly used as a plasticizer. It is a ubiquitous environmental contaminant. It was listed as priority pollutant by US Environmental Protection Agency. The present study aimed at determining the acute toxicity (LC_{50} Value) and histopathological impact of DnBP on *C.carassius.* The acute toxicity assay was carried out according to the standard methods in APHA and the value was assessed using the Probit Analysis method. The fish models were acclimatized to the laboratory conditions for a period of 14 days. The stock solution of DnBP was prepared and fishes were exposed to various doses ranging from 2-22 ppm for 96hrs. The result confirmed that the median lethal dose (LC_{50}) of DnBP for the fish, *Carassius carassius* is 7.77 ppm. Signs of abnormal behavior were also noticed during the test such as loss of equilibrium, erratic swimming, lethargy and motionlessness. The study concluded that DnBP is highly toxic to fish, *Carassius carassius* with the evidence of behavioral deformations. Several degenerative changes in the histology of liver and gill tissues exposed to DnBP were noticed. Liver showed various circulatory deformities (hyperaemia, blood congestion and sinusoidal dilatation) and vacuolization of hepatocytes, whereas Gills showed hyperaemia, epithelial lifting, oedema, talengiectasia, epithelial hyperplasia and fusion of secondary lamellae. Our study concluded that exposure of *C.carassius* to DnBP results in tissue morphological changes. The study also described that the gills were more affected than the liver probably because of their direct contact with DnBP.

Keywords- DnBP, Acute toxicity, LC_{50}, Probit analysis, Histopathology, *C.carassius.*

1. Introduction

Fishes have significant economic importance and are quite sensitive to the wide array of pollutants discharged in the aquatic ecosystems. Fishes are widely used to evaluate water standard of aquatic environment because they serve as pollution bioindicators and play notable roles in assessing potential risk associated with contamination of new chemicals in aquatic ecosystem (Lakra and Nagpure, 2009). In Kashmir Cyprinids are the most notable family of fish, and its members are distributed globally. These

family members are distributed broadly in fresh water sources (Demirsoy, 1988, Geldiay and Balik, 1998). Freshwater Cyprinid fish dominates global aquaculture production. Some characteristics of *C. carassius* L. (Cyprinidae) such as its wide distribution and availability throughout the year, cost-effectiveness, easy handling and acclimatization in the laboratory make it an excellent ecotoxicological model.

Environmental pollutants, like xenobiotic substances released as byproducts of anthropogenic actions, naturally lead to pollution of aquatic environments. They negatively affect the environment through unfavourable impacts on growth, development and reproduction of aquatic organisms (Johnson and Yund, 2007), lead to a keen fall in number besides quality of the aquatic population (Reynolds et al, 2005). As a downstream impact, such pollution also affects human and animal health chiefly in cases where fish is consumed or utilized as a food source. This is because fish are common pollutant bioaccumulators and have the highest potency for transferring such residues to humans (Dorea, 2006). One of the outstanding examples of xenobiotics is endocrine disrupting compounds (EDCs) such as phthalate esters (PEs), which have the efficacy to disturb numerous biological systems including the invertebrate, reptilian, avian, aquatic and also the mammalian systems (Moder et al, 2007)

Phthalates are family of xenobiotic hazardous compounds amalgamating in plastics to intensify their plasticity, flexibility, longevity, versality and durability. Besides they are also used as lubricants, solvents, additives, softeners etc. They are present in number of day to day used products such as PVC products, building materials (paint, adhesive, wall covering), personal-care products (perfume, eye shadow, moisturizer, nail polish, deodorizer etc), medical devices, detergents and surfactants, packaging, children's toys, pharmaceuticals and food products, textiles, household applications such as shower curtains, floor tiles, food containers and wrappers etc. They are ubiquitous environmental contaminants entering environment via various routes. Once entering the environment, they pose remarkable toxicological threats to the myriad of non target organisms, discover its way to the food chain, and threaten ecological balance and biodiversity of nature. The effluents generated from waste water treatment plants have been considered as main source of plasticizers in aquatic environment (Loraine and Pettigrove, 2006). Due to potential risk of phthalates for organism's health and environment, a number of them have been incorporated in the priority pollutant list of several national and supranational federations.

Di-n-butyl phthalate(DnBP) is one of the commonly used phthalate essentially as plasticizer to ameliorate the flexibility and workability of the products, such as polyvinyl chloride, plastic packaging films, adhesives, lubricants, cellulose materials, cosmetics and insecticides (Gao and Wen, 2016). DnBP is not chemically attached to the polymer matrix like other phthalates, directing to its ubiquitous existence in the diverse environmental matrices (Net et al, 2015). DnBP has been directly assessed for reproductive and developmental toxicity in addition to the monitoring of testicular germ cell toxicity and testicular atrophy in standard estimation (Oishi and Hiraga, 1980, Gray et al. 1982, Barber et al. 1987, Srivastava et al.1990). DnBP is considered very dangerous substances in the EU REACH regulation and is classified as category

1B in the Commission Directive 2007/19/EC (cannot be used to make toys, childcare articles, and cosmetics) and risk reduction measures are required for its safe use. Canada and the United States have also taken regulatory actions restricting their use (Ventrice et al. 2013). Furthermore, it poses a particular risk to aquaculture.

Toxicity tests have been performed on fishes to estimate the effect of toxins on various aquatic organisms under laboratory conditions. The 96-h acute toxicity, described as median lethal concentration (LC_{50}) value is contemplated appropriate for toxicological testing and safety assessment of the organic chemicals. The LC_{50} value of a chemical is defined as its concentration in water that kills 50% batch of test animal (fish in this study) within a continuous period of exposure which must be stated.

Histopathological investigation is very subtle aspect and is very pivotal in assessing cellular changes that might occur in target organs, such as the liver, gut, kidney, gills etc. Histopathological study is important to notice the infection and the nature of relationship of clinical signs. Histopathological investigations have long been perceived to be valid biomarkers of stress in fish (Van der Oost et al, 2003). Histopathological alterations have been broadly used as biomarkers in the assessing of the health of fish exposed to contaminants. One of the significant advantage of using histopathological biomarkers in the observing of environment is that this class of biomarkers permits investigating specific target organs including kidney, liver and gills, that are accountable for crucial functions, such as respiration, excretion and accumulation and biotransformation of xenobiotics in the fish (Gernhofer et al, 2001). Moreover, the changes in these organs are generally easier to recognize than functional ones (Fanta et al, 2003) and serve as alert signs of damage to animal health (Hinton and Lauren, 1990). The present study aimed at determining the acute toxicity, genotoxicity and histopathological impact of DnBP on *C.carassius.*

2 .Materials and methods

2.1 Chemicals and reagents

The chemicals used in the current study were of high clarity. Di-n-butyl phthalate ($C_{16}H_{22}O_4$, DnBP, CAS No. 84-74-2, 99% purity) was procured from Sigma- Aldrich; Bengaluru, India is a colorless to faint yellow viscous liquid. Acetone, $(CH_3)_2C0$, CAS No.67-64-5, 99% , formalin , paraffin wax, hematoxylin , eosin etc were purchased from Hi- Media Labs, Mumbai, India and are 99.95% of purity.

2.2 Test organism

C. carassius L. (Family: Cyprinidae and Order: Cypriniformes was selected as the experimental model. Locally known as “Gang Gad”, it is a freshwater fish occurring in the standing and slow flowing waters, especially the flat land lakes of the Kashmir Valley. Live juvenile fish were procured with the help of a local fisherman, using hand nets, from the Dal Lake (34°07′N 74°52′E), in the vicinity of the University of

Kashmir, Srinagar, India. They were transported slive in plastic jars to the Cytogenetics and Molecular Biology Laboratory, Centre of Research for Development (CORD), University of Kashmir and subjected to a prophylactic treatment by bathing in a 0.05 % aqueous solution of potassium permanganate for 2 m to avoid dermal infection. Their average length and wet weight (± SD) were recorded as 12.5 ± 1.64 cm and 33 ± 4.94 g, respectively.

2.3 Acclimatization

The fish stock was acclimatized before the commencement of the experiment for at least 3 weeks to a 1:1 diurnal photoperiod in well aerated 60 L glass aquaria at 19.7 ± 2.6°C with 24 h aged dechlorinated tap water (pH 7.6 – 8.4) and fed daily with commercially available fish food (Feed Royal®, Maa Agro Foods, Visakhapatnam, Andhra Pradesh, India). Only active specimen with no sign of injury and distress were used in the study. Waste products were siphoned off every day to check increase of ammonia in the water. Every effort as suggested by Bennett and Dooley (1982) was taken to maintain optimal conditions during acclimatization: no fish died during this period. The acclimatized fish were used for the experiments. Studies involving experimental animals were conducted in accordance with the guidelines described for maintenance, care and conducting toxicity tests of fish in Standard Methods for the Examination of Water and Wastewater, American Public Health Association (APHA, AWWA and WPCF, 2005).

2.4 Median lethal concentration assessment (96-h LC_{50}) and toxicity symptoms of DnBP in juvenile fish

Determination of 96 h- LC_{50} of DnBP to *C. carassius* was conducted in a semi-static system with 60 L glass aquaria, changing the DnBP solution after every 24 h to maintain similar concentration throughout the experiment. Following range finding tests, fishes were exposed to eleven different concentrations of DnBP (2,4,6,8,10,12,14,16,18,20,22 ppm). Each group was assayed in duplicate. Blank controls one of tap water and another of acetone were also included in experiment. Each test group was exposed to varying concentrations of DnBP and the resultant mortalities were counted and recorded at 24, 48, 72 and 96 h intervals. No food was provided to the fish throughout the experiment and lethality was the toxicity end-point. Fish were visually examined daily and considered dead when no respiratory movements or no sudden swimming in response to gentle touching were observed. Dead fish were gently and immediately removed from the aquaria. During the acute toxicity testing, fishes were examined for abnormal behaviour and external appearance.

2.4.1 Statistical Analysis

Finally to find out the concentration of DnBP at which 50% mortality of fish could occur, the Probit Statistical Analysis (Finney,1971) was done using SPSS statistical analysis software (24.0) and LC_{50} was

determined with (95% confidence limit). Moreover to make analysis conducive regression equation (y=mortality and X=concentration) was found out, the LC_{50} was derived from the best-fit line obtained.

2.5 Experimental design for histopathological examination

Standardized OECD testing guidelines were followed for semi- static bioassay changing DnBP solution after every 24hrs for 96 hrs. The acclimatized fish specimens were divided into two groups each of 10 fishes. Group (I) fish were kept as control (no treatment was given) and group (II) fish were subjected to ½ 96h-LC_{50} (3.88 mg/L) for 96 hrs.

Towards the end of exposure period, 5 fish were taken from Both Aquaria. The gill arches of the fish were removed from both sides. Fish were dissected, the abdominal cavity was operated and the liver was removed quickly and was fixed in 10% Formalin as a histological fixative for 24 h. According to Humason (1967), the specimens were processed as usual in the recognized method of dehydration, cleared in xylene and finally embedded in paraffin wax before being sectioned at 5 µm using a rotary microtome. The specimens were stained with Hematoxylin and Eosin. Finally, the prepared sections were examined and photographically enlarged using light microscopy.

3. Results

3.1 LC_{50} value and clinical observations

The calculated 96-h LC_{50} value (with 95% confidence limit) for DnBP, using a semi static bioassay system on *C. carassius* was found to be 7.77 mg/L as shown in Table 1.The relation between percent mortality rate and the concentration of DnBP have been drawn according to Finney's Probit analysis using SPSS statistical analysis software (24.0). Figure-1 shows the regression line between the mortality of *C. carassius* and log concentration of DnBP. It was observed that a dose-dependent increase and time –dependent decrease occurs in mortality rate such that as the exposure time increases from 24h to 96h, the median lethal concentration required for killing the fish was reduced. No mortality was observed in control groups during whole experiment.

Table 1: **96h-LC_{50} value of DnBP to *C.carassius.***

Compound	LC_{50} (mg/L)	Regression equation	Correlation coefficient (R^2)	Standard Error
DnBP	7.77	Y =2.682x + 2.613	O.95	0.22

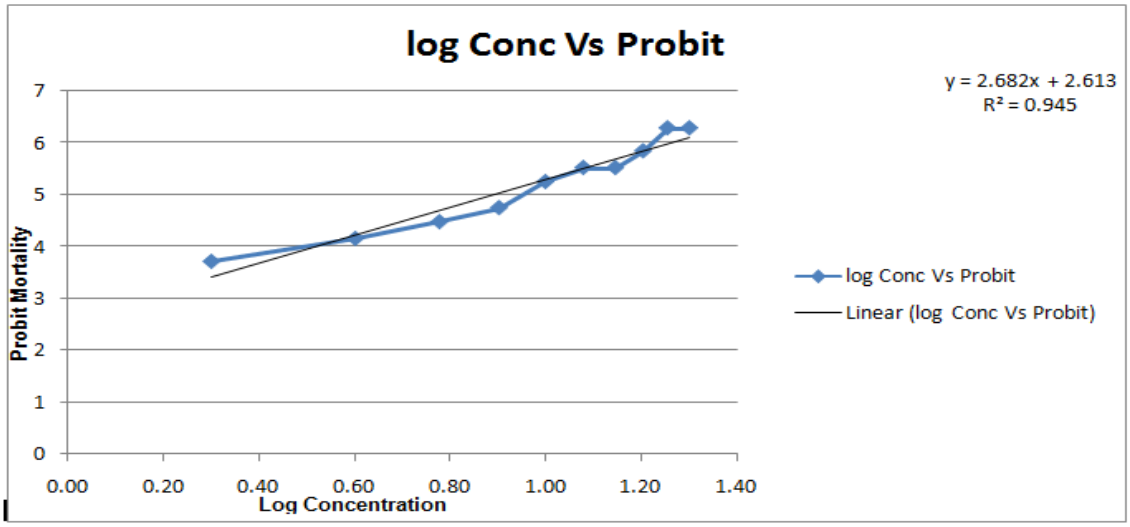

Figure-1. Regression line showing positive correlation between probit mortality of *C.carassius* and log concentration of DnBP

During acute toxicity assay no clinical signs were noticed throughout the period of exposure in control groups. However, within 8h of exposure to DnBP, Carps in each group showed different intoxications symptoms. In the higher concentration groups, fishes were fully evoked shortly after contacting the solution contrast to lower concentration groups, but the intoxication characters were same as in higher concentration groups. Abnormal behaviour was noted immediately such as loss of equilibrium, erratic swimming movements, lethargy and motionlessness followed by convulsions (table 2). The fish under experimental study exhibited difficulty in breathing represented by speedy breathing coexisting with rapid movement of operculum and failure to respond to escape reflex. Furthermore dark discoloration of skin with thick layer of mucous was also noted. Postmortem studies revealed congestion of internal organs and excessive slime deposition on gills.

Table 2.The behavioral changes of *C.carassius* exposed to different concentrations of DnBP for 96hrs.

Behavioural changes	2mg/L[a]	4mg/L[a]	6mg/L	8mg/L	10mg/L	12mg/L	14mg/L	16mg/L	18mg/l	20mg/L	22mg/L[b]
Erratic Swimming	–	–	+	+	+	+	+	+	+	+	-
Loss of equillbrum	–	–	_	+	+	+	+	+	+	+	-
Became lethargic	–	–	_	+	+	+	+	+	+	+	-
Motionlessness	–	–	_	_	_	+	+	+	+	+	-
Convulsions	–	–	_	_	_	_	+	+	+	+	-

(+):Behavoioural abnormalities were observed; (-): behavioural abnormalities were not observed.

[a]The abnormalities were not noticed because all the fishes were not affected by the toxicant.

[b]The abnormalities were not noticed because all the fishes are death .

3.2 Histopathological findings

The present study reports the histopathological impact of sublethal DnBP on gills and liver of *C.carassius*. Fish in the control groups displayed no histopathological alteration in the examined tissues. Nevertheless, exposure to sublethal DnBP resulted in degenerative changes in the gills and liver.

Gills

The principal alterations found in gill tissue of *C.carassius* exposed to sublethal DnBP for 96h were epithelial lifting, oedema, hyperaemia talengiectasia, epithelial hyperplasia and fusion of secondary lamellae (Fig. 2).

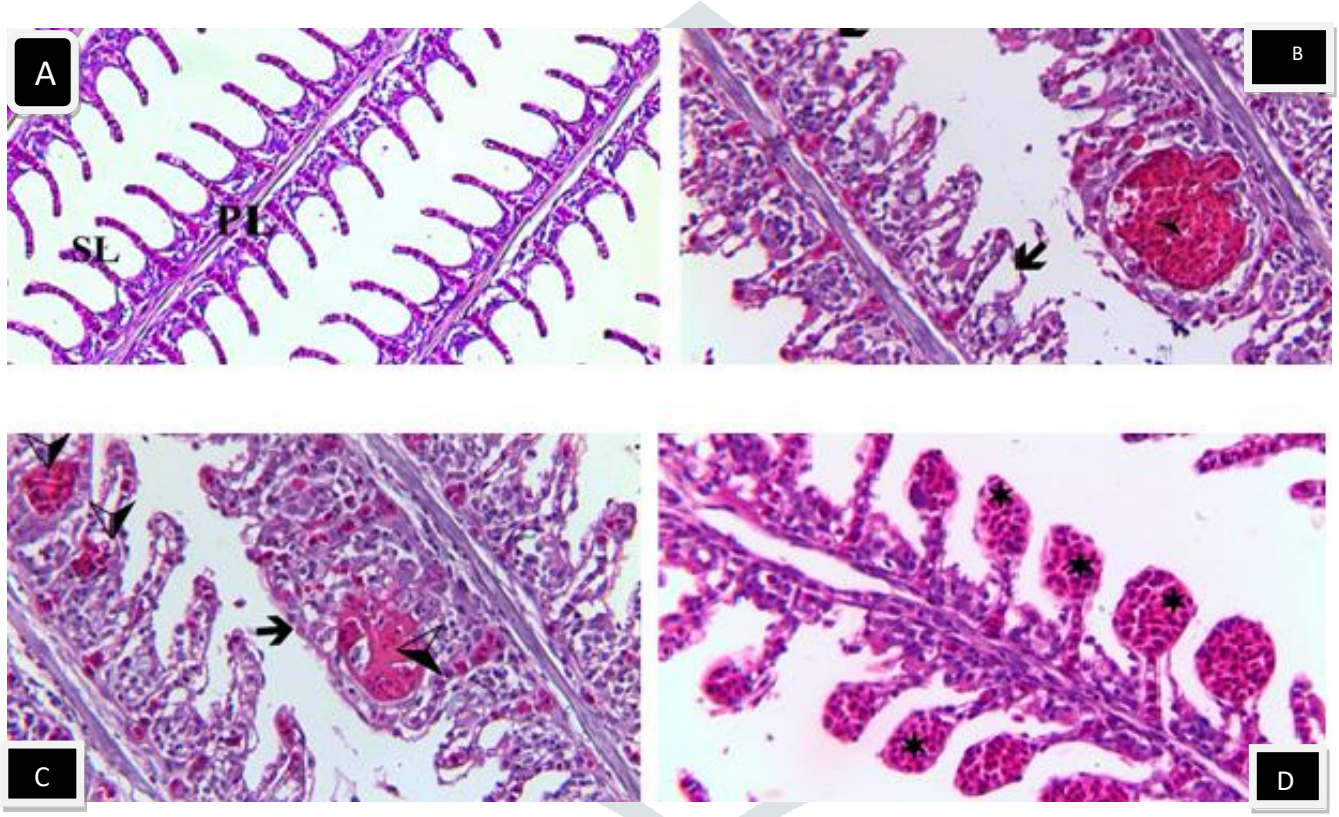

Figure 2: Histological aspects of the gill tissue (A) Gill architecture normal in control fish. PL, primary lamellae;SL, secondary lamellae (B) Hyperaemia (arrowhead) and epithelial lifting and oedema (arrows) observed in gill after 96h DnBP exposure (C) Lamellar fusion (arrow) and hyperaemia (arrowheads) in the gills of fish exposed for 96 h to DnBP (D) Telangiectasis (asterisks) observed in gill after 96 h DnBP exposure

Liver

Numerous circulatory deformities (blood congestion and hyperaemia, sinusoid dilatation) and vacuolization of hepatocytes (Fig. 3) were observed after 96h exposure

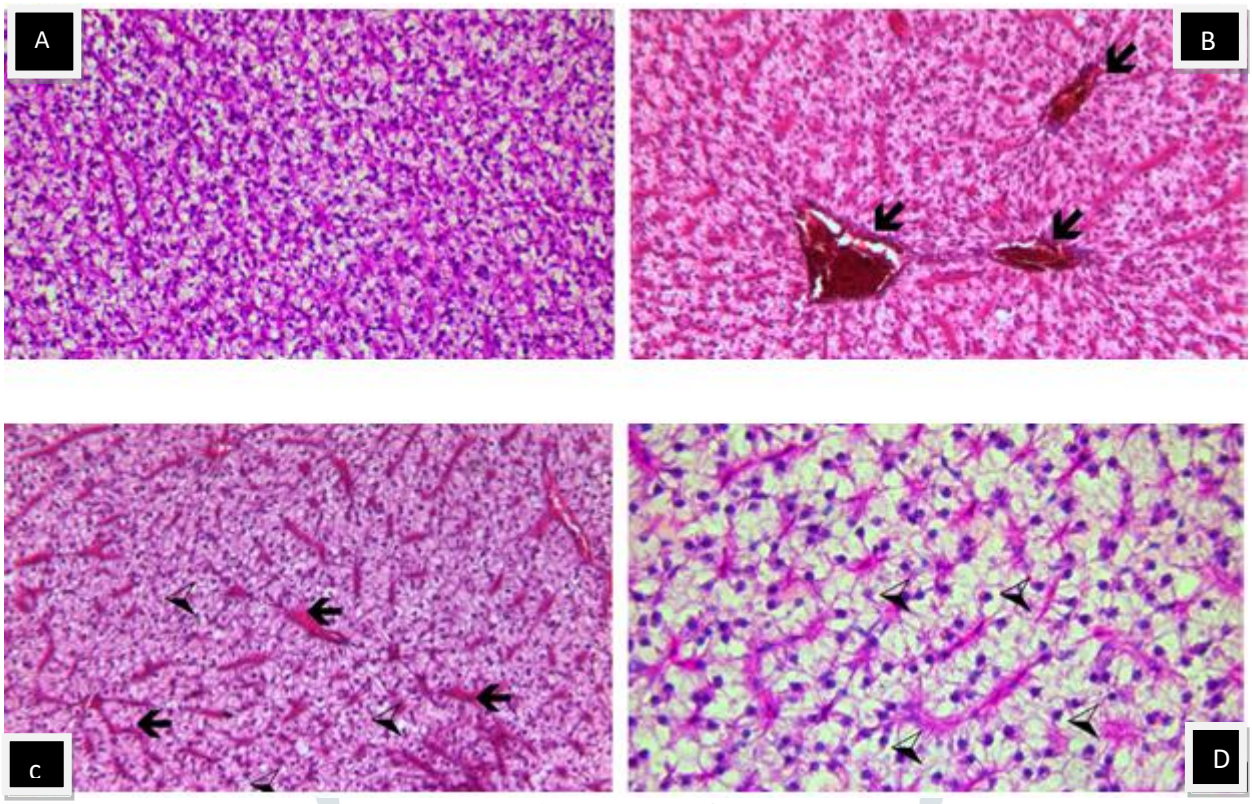

Figure 3: Histological appearance of the liver tissue (A) Liver architecture normal in control fish (B) Passive hyperaemia (arrows) observed in liver after 96 h DBP exposure (C) Passive hyperaemia (arrows) and hydrophic vacuolization (arrowheads) in the liver of fish exposed for 96 h to DBP (D) Hydrophic vacuolization (arrowheads observed in liver after 96 h DBP exposure .

4. Discussion

At present, numerous chemicals have been classified as plasticizers and studies using different models have indicated that some of them have toxic properties. Pollution of aquatic environment due to plastic residues is well documented and fish are often used as sentinel organisms for eco-toxicological studies as they are able to accumulate genotoxic substances and respond to low concentration of mutagens in a manner similar to higher vertebrates (Spitsbergen and Kent, 2003, Cavas et al. 2005). Therefore, the use of fish biomarkers as indices of the effects of pollution, are of great importance, and help in early detection of aquatic environmental problems (Van Der oost et al. 2003).

In the present study, pre-treatment of (0.05 %) solution of potassium permanganate was given to the fish for 2 min to avoid any dermal infection and after that the specimen were acclimatized for at least 3 weeks under laboratory conditions to remove the residual effects of other chemicals prior to start of the experiment. Several investigators (Pandey et al. 2006, Sharma et al. 2007, Ali et al. 2009) have used potassium permanganate solution for prophylactic treatment before starting their experiments, and like our study, they did not report any adverse effects in the test organism due to prophylactic treatment.

A number of studies have been published on DnBP toxicity in early stages of aquatic species (Xu et al. 2013a, Xu et al. 2013b) but reports with regard to its toxicity using LC_{50} fractions are relatively sparse. In an attempt to fill this lacuna, the study was conducted to assess the 96h- LC_{50} of DnBP in *C.carassius* using Probit Analysis. The Probit analysis is commonly used in toxicological studies to determine relative toxicity of chemicals to living organisms. In present study probit analysis has been done by drawing regression line between probit kill of *C.carassius* and log of concentration of DnBP. Finally, the calculated 96h- LC_{50} of DnBP for *C.carassius*, as obtained based on Probit Analysis, was 7.77mg/L. The subsequent data indicated that DnBP at the given concentration is highly toxic to *C.carrassius*. Several acute toxicity studies have been reported for 96 h -LC_{50} values in case of fish species other than *C.carassius*; the reported 96h-LC_{50} value of DnBP in Nile tilapia (*Oreochromis niloticus)* was 11.8mg/L (Khalil et al, 2016) Also, in a study, Zhao et al. (2014) reported that the DnBP 96-h LC_{50} in case of carp (*Cyprinus carpio*) is 16.30 mg/L. The noticed variation in the sensitivity of fish to DnBP can be accounted for by differences in kinetic parameters, species, size, age, health as well as experimental conditions (Eaton and Gilbert, 2008). The valuable scientific data drawn from acute toxicity studies was acquired from a combination of behavioral, clinical and postmortem observation of test animals in addition to the LC_{50} value (Eaton and Gilbert, 2008). The clinical alterations observed in the test subjects exhibited as perturbations in their respiratory and movement patterns and seemed to appear almost immediately after exposure to high DnBP concentrations, where these behavioral deviations became more pronounced as DnBP levels were increased. The altered respiratory pattern may be a byproduct of post-stress related excessive mucus secretion which results in the formation of a thick coat on the gill tissue which causes irritation to the gills Behavior-related alterations observed in our study are hypothesized to be a strategy by which the animals adapt to changes in the surrounding environment upon exposure to pollutants. The study on fishes is of great importance to provide a future understanding of ecological impact.

The hyperplasic alterations (epithelial hyperplasia and lamellar fusion) are defence responses which have been reported to safeguard the organism due to the decreased uptake of the chemical by increasing distance between the toxicant and blood vessels (Mallatt, 1985, Reiser et al. 2010, Xu et al. 2014). Fish gills are mainly contemplated as best indicators of toxicant exposure due to the direct interaction with the surrounding medium and their vulnerable structure (Mallatt, 1985, Poulino et al. 2012). In our study, most of the changes detected in gills are common in numerous fish species exposed to various different kinds of pollutants (Benli et al. 2008, Sepici-Dinc et al. 2009, Poulino et al. 2012, Agamy, 2013). Nevertheless, studies related histological impacts of phthalate on gill histology in fish are sparse. According to Xu et al. (2014) and Chen et al. (2015), DnBP is probably efficiently absorbed by the gills Because of its high lipophilicity. Xu et al. (2014) described lamellar fusion epithelial hyperplasia and oedema in adult male Zebrafish after 45 days exposure to 100 and 500 µg /L DnBP. Alike results were described by Chen et al.

(2015) in a study with sublethal concentrations of the DnBP (0.1 and 0.5 mg /L) on Zebrafish after 90 days post fertilization

According to Hinton and Lauren (1990), hepatocytes vacuolization is related with the inhibition of protein synthesis and energy depletion and is a principal response to various chemical stressors (Liao et al, 2006). Fish liver is the chief organ of numerous metabolic pathways and alteration in liver histology is presently broadly used as biomarkers of toxicant and carcinogen exposure (Benli et al. 2008, Sepici-Dinc et al. 2009, Boran et al. 2012, Munoz et al. 2015). Xu et al. (2014) reported cloudy swelling, pyknosis, vacuolization and accumulation of lipid droplets in adult male Zebrafish exposed to 100 and 500 µg /L DnBP after 45 days supporting results of this study. Nevertheless, Chen et al. (2015) did not find any notable change in the liver of Zebrafish exposed to sublethal DnBP after 90 days post fertilization. They described that exposure to 20 ng /L Ethynylestradiol (EE2) in combination with DnBP (0.1 msg /L or 0.5 mg/ L) resulted in vacuolization of the liver.

5. Conclusion

The study on fishes is of great importance to provide a future understanding of ecological impact. The present study was an attempt to find the acute toxicity and histopathological impact of DBP on *C.carassius.* The results of acute toxicity exclusively showed that administration of DBP induced mortalities in model organism *C.carassius* at various concentrations confirming its acute toxic potential. The median lethal concentration (96 h-$LC_{50)}$ of DBP was 7.77mg/L, thus confirms that it belongs to high toxic level compounds. The results of histopathological examination of gills and liver after sublethal exposure to DnBP showed alterations in both tissues. Therefore, we suggest that the histopathological changes of certain target tissues acts as biomarkers of environmental exposure of freshwater fish to DnBP. Additional studies are needed to shed light on chronic exposure of DnnBP and evidence of repair during period following a DBP exposure.

6. Acknowledgement

Authors are highly thankful to the Centre of Research for Development (CORD), University of Kashmir for providing laboratory facilities to carryout present research work.

References

[1] Agamy, E. 2013. Impact of laboratory exposure to light Arabian crude oil, dispersed oil and dispersant on the gills of the juvenile brown spotted grouper (Epinephelus chlorostigma): A histopathological study. Marine Environmental Research. 86: 46-55.

[2] Ali, D., Nagpure, N.S., Kumar, S., Kumar, R., Kushwaha, B. and Lakra, W.S. 2009. Assessment of genotoxic and mutagenic effects of Chlorpyrifos in freshwater fish Channa punctatus (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Food and Chemical Toxicology.47 (3), 650-656.

[3] APHA, AWWA, WPCF, 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Publication of Health Association, Washington, DC.

[4] Barber, E. D., Astill, B. D., Moran, E. J., Schneider, B. F., Gray, T. J., Lake, B. G. and Evans, J. G. 1987. Peroxisome induction studies on seven phthalate esters. Toxicology and industrial health, 3(2): 7-24.

[5] Benli, A.Ç. K., Köksal, G. and Özkul, A. 2008. Sublethal ammonia exposure of Nile tilapia (Oreochromis niloticus L.): Effects on gill, liver and kidney histology. Chemosphere. 72(9): 1355-1358.

[6] Bennett, R.O. and Dooley, J.K. 1982. Copper uptake by two sympatric species of Killifish: Fundulus heteroclitus (L.) and F. majalis (Walbaum). Journal of Fish Biology. 21(4), 381-398.

[7] Boran, H., Capkin, E., Altinok, I. and Terzi, E. 2012. Assessment of acute toxicity and histopathology of the fungicide captan in rainbow trout. Experimental and Toxicologic Pathology. 64(3): 175-179.

[8] Cavas, T., Garanko, N.N. and Arkhipchuk, V.V. 2005. Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate. Food and Chemical Toxicology. 43(4), 569-574.

[9] Chen, P., Li, S., Liu, L. and Xu, N. 2015. Long-term effects of binary mixtures of 17α-ethinylestradiol and dibutyl phthalate in a partial life-cycle test with Zebrafish (Danio rerio). Environmental Toxicology and Chemistry. 34(3): 518-526.

[10] Demirsoy, A. 1988. Yasamin Tamel Kurallari. Hacettepe Universitesi Yayinlari. Ankara, Turkey, 684p.

[11] Dorea, J. G. 2006. Fish meal in animal feed and human exposure to persistent bioaccumulative and toxic substances. Journal of Food Protection. 69(11): 2777-2785.

[12] Eaton, L. D. and Gilbert, S. G. 2008. Principles of toxicology.In: Casarett and Doull's Toxicology: the basic science of poisons, 7th ed.pp.11-44. Klassen , C.D. ed., New York: McGraw-Hill

[13] Fanta, E., Rios, F. S. A., Romão, S., Vianna, A. C. C. and Freiberger, S. 2003. Histopathology of the fish Corydoras paleatus contaminated with sublethal levels of organophosphorus in water and food. Ecotoxicology and Environmental Safety. 54(2): 119-130.

[14] Finney, D.J., 1971. Probit Analysis, Cambridge University Press,Cambridege ,United Kingdom

[15] Gao, D. W. and Wen, Z. D. 2016. Phthalate esters in the environment: A critical review of their occurrence, biodegradation, and removal during wastewater treatment processes. Science of the total Environment, 541: 986-1001.

[16] Gernhofer, M., Pawert, M.,Schramm, M., Müller, E. and Triebskorn, R. 2001. Ultrastructural biomarkers as tools to characterize the health status of fish in contaminated streams. Journal of Aquatic Ecosystem Stress and Recovery. 8(3-4): 241-260.

[17] Gray, T. J. B., Rowland, I. R., Foster, P. M. D. and Gangolli, S. D. 1982. Species differences in the testicular toxicity of phthalate esters. Toxicology letters, 11(1-2): 141-147.

[18] Hinton D.E. and Lauren D.J. 1990. Integrative histopathological approaches to detecting effects of environmental stressors in fishes. Proceedings of the American Fisheries Society Symposium. 8: 51–66.

[19] Humason G.L. 1967. Animal tissue technique. Freemand, W.H. and Co.Sanfrancisco

[20] Johnson, S.L. and Yund, P.O. 2007. Variation in multiple paternity in natural populations of a free-spawning marine invertebrate. Molecular Ecology. 16(15): 3253-3262.

[21] Khalil, S.R., Elhakim, Y.A. and EL-Murr A.E. 2016. Sublethal concentrations of di-n-butyl phthalate promote biochemical changes and DNA damage in juvenile Nile tilapia (Oreochromis niloticus). Japanese Journal of Veterinary Research. 64(1), 67-80.

[22] Lakra, W. S. and Nagpure, N. S.2009. Genotoxicological studies in fishes: a review. Indian Journal of Animal Sciences, 79(1): 93-97.

[23] Liao, C. Y., Fu, J. J., Shi, J. B., Zhou, Q. F., Yuan, C. G. and Jiang, G. B. 2006. Methylmercury accumulation, histopathology effects, and cholinesterase activity alterations in medaka (Oryzias latipes)

following sublethal exposure to methylmercury chloride. Environmental Toxicology and Pharmacology. 22(2): 225-233.

[24] Loraine, G. A. and Pettigrove, M. E. 2006. Seasonal variations in concentrations of pharmaceuticals and personal care products in drinking water and reclaimed wastewater in southern California. Environmental Science and Technology. 40(3): 687-695.

[25] Mallatt, J. 1985. Fish gill structural changes induced by toxicants and other irritants: a statistical review. Canadian Journal of Fisheries and Aquatic Sciences. 42(4): 630-648.

[26] Moder, M., Braun, P., Lange, F., Schrader, S. and Lorenz, W. 2007. Determination of Endocrine Disrupting Compounds and Acidic Drugs in Water by Coupling of Derivatization, Gas Chromatography and Negative-Chemical Ionization Mass Spectrometry. Clean–Soil, Air, Water. 35(5): 444-451.

[27] Munoz, L., Weber, P., Dressler, V., Baldisserotto, B. and Vigliano, F. A. 2015. Histopathological biomarkers in juvenile silver catfish (Rhamdia quelen) exposed to a sublethal lead concentration. Ecotoxicology and Environmental Safety. 113: 241-247.

[28] Net, S., Rabodonirina, S., Sghaier, R. B., Dumoulin, D., Chbib, C., Tlili, I. and Ouddane, B. 2015. Distribution of phthalates, pesticides and drug residues in the dissolved, particulate and sedimentary phases from transboundary rivers (France–Belgium). Science of the Total Environment, 521: 152-159.

[29] Oishi, S. and Hiraga, K. 1980. Testicular atrophy induced by phthalic acid esters: effect on testosterone and zinc concentrations. Toxicology and applied pharmacology, 53(1): 35-41.

[30] Pandey, S., Nagpure, N. S., Kumar, R., Sharma, S., Srivastava, S. K. and Verma, M. S. 2006. Genotoxicity evaluation of acute doses of endosulfan to freshwater teleost Channa punctatus (Bloch) by alkaline single-cell gel electrophoresis. Ecotoxicology and environmental safety, 65(1): 56-61.

[31] Poulino, M. G., Souza, N. E. S. and Fernandes, M. N. 2012. Subchronic exposure to atrazine induces biochemical and histopathological changes in the gills of a Neotropical freshwater fish, Prochilodus lineatus. Ecotoxicology and Environmental Safety. 80 : 6-13.

[32] Reiser, S., Schroeder, J. P., Wuertz, S., Kloas, W. and Hanel, R. 2010. Histological and physiological alterations in juvenile turbot (Psetta maxima, L.) exposed to sublethal concentrations of ozone-produced oxidants in ozonated seawater. Aquaculture. 307(1-2): 157-164.

[33] Reynolds, J. D., Dulvy, N. K., Goodwin, N. B. and Hutchings, J. A. 2005. Biology of extinction risk in marine fishes. Proceedings of the Royal Society of London B: Biological Sciences. 272(1579): 2337-2344.

[34] Sepici-Dinçel, A., Benli, A. Ç. K., Selvi, M., Sarıkaya, R., Şahin, D., Özkul, I. A. and Erkoç, F. 2009. Sublethal cyfluthrin toxicity to carp (Cyprinus carpio L.) fingerlings: biochemical, hematological, histopathological alterations. Ecotoxicology and Environmental Safety. 72(5): 1433-1439.

[35] Sharma, S., Nagpure, N.S., Kumar, R., Pandey, S., Srivastava, S.K., Singh, P.J. and Mathur, P.K.2007. Studies on the genotoxicity of endosulfan in different tissues of fresh water fish Mystus vittatus using the comet assay. Archives of Environmental Contamination and Toxicology. 53(4), 617-623.

[36] Spitsbergen, J.M. and Kent, M.L. 2003. The state of the art of the Zebrafish model for toxicology and Toxicologic pathology research—advantages and current limitations. Toxicologic Pathology, 31(1_suppl), 62-87.

[37] Srivastava, S. P., Srivastava, S., Saxena, D. K., Chandra, S. V. and Seth, P. K. 1990. Testicular effects of di-n-butyl phthalate (DBP): biochemical and histopathological alterations. Archives of toxicology, 64(2): 148-152.

[38] Van der Oost, R., Beyer, J. and Vermeulen, N. P. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology. 13(2): 57-149.

[39] Ventrice, P., Ventrice, D., Russo, E. and De Sarro, G. 2013. Phthalates: European regulation, chemistry, pharmacokinetic and related toxicity. Environmental toxicology and pharmacology, 36(1): 88-96.

[40] Xu, H., Shao, X., Zhang, Z., Zou, Y., Chen, Y., Han, S. andChen, Z. 2013a. Effects of di-n-butyl phthalate and diethyl phthalate on acetyl cholinesterase activity and neurotoxicity related gene expression in embryonic Zebrafish. Bulletin of Environmental Contamination and Toxicology. 91(6), 635-639.

[41] Xu, H., Shao, X., Zhang, Z., Zou, Y., Wu, X. and Yang, L. 2013b. Oxidative stress and immune related gene expression following exposure to di-n-butyl phthalate and diethyl phthalate in Zebrafish embryos. Ecotoxicology and Environmental Safety. 93, 39-44.

[42] Xu, N., Chen, P., Liu, L., Zeng, Y., Zhou, H. and Li, S. 2014. Effects of combined exposure to 17α-ethynylestradiol and dibutyl phthalate on the growth and reproduction of adult male zebrafish (Danio rerio). Ecotoxicology and Environmental Safety. 107: 61-70.

[43] Zhao, X., Gao, Y. and Qi, M. 2014. Toxicity of phthalate esters exposure to carp (Cyprinus carpio) and antioxidant response by biomarker. Ecotoxicology. 23(4), 626-632.