International Journal of Zoological Investigations

Contents available at Journals Home Page: www.ijzi.net

ISSN: 2454-3055

Sublethal Toxic Effects of Bisphenol A on Oxygen Consumption, Haematological and Histological Parameters in the Cichlid Fish, *Pseudetroplus maculatus* (Bloch, 1795)

Asifa K. P. and Chitra K. C.*

Endocrinology and Toxicology Laboratory, Department of Zoology, University of Calicut, Malappuram District, Kerala, 673 635, India

**Corresponding Author*

Received: 2nd April 2018
Accepted: 29th April 2018

Abstract: The cichlid fish *Pseudetroplus maculatus* was used to evaluate the sublethal toxic effects of estrogenic environmental contaminant, bisphenol A. Fish were exposed to sublethal concentration (648 µg/L) of bisphenol A for short-term (24, 72 and 96 h) and long-term (7 and 15 days) durations along with control groups. The rate of oxygen consumption was found to be decreased as the treatment period increases and this could be due to the accumulation of mucous on gills or shrinkage of the respiratory epithelium as an outcome of contaminant toxicity. Haematological parameter such as erythrocyte count was decreased while total leukocyte count increased significantly in time-dependant manner. The significant decrease in erythrocyte count indicates destruction of RBC or reduction in erythropoietic activity as a result of bisphenol A exposure. Increase in total leukocyte count is a sign of hypersensitivity that convey immunological reactions to produce antibodies in order to overcome the stress. Histological examination revealed that bisphenol A exposure caused upliftment of gill epithelium, hyperplasia of gill arches, aneurysm and absence of secondary lamellae. Hepatic cells showed vacuolization and degeneration of cytoplasm after the exposure to bisphenol A when compared to the control tissues. Thus the results of the present study clearly demonstrate that exposure of bisphenol A at sublethal concentration to the fish may provoke adverse effects on vital organs.

Keywords: Bisphenol A, *Pseudetroplus maculatus*, Oxygen consumption, Haematology, Histology, Gill, Liver

Introduction

In recent years, pollution of water resources has become a serious problem which leads to several ecological disorders and many physiological as well as biological changes in aquatic animals. Industrial and agricultural developments, urbanization and increased population growth are the major causes of contamination of water resources. Indiscriminate use of pesticides, dumping of industrial waste and release of domestic

sewage directly into freshwater resources are some of the factors that diverse the normal life forms of the aquatic habitats. Thus large number of aquatic animals, especially fish species, has failed to cope up with the environmental stress caused by increased concentrations of different chemicals that are discharged into the water bodies. In aquatic environments, the concentration of contaminants may vary and usually, it may be at lethal or sublethal levels. Lethal concentration of a chemical causes death of the organisms directly but the sub-lethal concentrations affect various physiological activities and disturb the metabolic processes of the exposed organism (Relyea and Hoverman, 2006). Acute toxicity tests are usually performed in different animals such as bacteria, fish or mammals in order to detect the hazardous effects of toxicants and biological dissociation tests have been carried out in detail to assess the effects of toxicants through the food cycle (Henschel *et al*., 1997). Evaluation of sublethal toxicity in fish acquire more attention because fishes have the tendency to bio-accumulate different xenobiotics since it occupy last position in aquatic food cycle and direct risks to humans through the food chain.

Since the fishes are completely engrossed in the surrounding water, they are highly sensitive to even a slight alteration in the external environment. Fishes have to pass large quantities of water over their respiratory surface continuously and are subjected to relatively greater risk of exposure to the toxic substances (Heath, 1995). In an aquatic environment, one of the most important manifestations of the chemical toxicant is overstimulation or depression of respiratory activity. Toxicant uptake of fish occurs mainly through the gills by simple diffusion, and thus the effects are well-documented through changes in gill membranes. Hence, the impact of toxicants on the respiration of fishes has received widespread attention by several researchers globally and the respiratory distress is considered as one of the earliest symptoms of acute toxicant exposure (McKim *et al*., 1987; David *et al*., 2015). In addition, alterations in the respiratory activity of fish have been used by several investigators as indicators of contaminant exposure. Oxygen consumption, an important physiological parameter is widely used to assess the toxic stress because it is a valuable indicator of energy expenditure in particular and metabolism in general. Several environmental contaminants are known to alter the rate of oxygen consumption in fish thereby indicate the possible impact of toxicants on the metabolic pathways (Yang *et al*., 2000).

Haematological examinations and analysis of serum constituents are the other widely used tests for the detection and diagnosis of metabolic disturbances and disease processes. Hence it is an important diagnostic tool for detecting the structural and functional status of fish exposed to toxicants. Blood parameters are highly sensitive and primarily employed for screening the health status of fish and other aquatic animals in the ecosystems (Blaxhall and Daisley, 1973). Thus, fish that lives in intimate contact with the environment are therefore highly vulnerable to physical and chemical changes, which may be reflected in the alterations in blood characteristics. In addition, environmental ecotoxicology and risk assessment use hematological parameters as

an important tool to assess the water quality in the field (Fazio *et al*., 2013).

Histopathological examination is the only semi-quantitative and sensitive tool to assess the impact of toxicant exposure by analyzing various signs, diseases and injury in cells, tissues, or organs (Arellano *et al*., 2001). Generally, fish tissues are sensitive indicators of aquatic pollution due to the bioaccumulation potential of exposed organic and inorganic compounds. Histopathological observations are the fast and valid method to diagnose the damages caused by various pollutants and several studies have reported that exposure to contaminants induce number of lesions and injuries to different organs of fish (Stentiford *et al*., 2003; Athikesavan *et al*., 2006). Usually, two types of structural changes have been identified in tissues as degeneration of tissues due to the direct toxic effect of the pollutant and necrosis of tissues as a result of compensatory mechanisms to cope up with the environmental stressors, which finally leads to cellular hyperplasia or hypertrophy (Hughes and Perry, 1976). Therefore, the objective of the present study is to assess the sublethal toxic effects of bisphenol A by assessing oxygen consumption, histological alterations in gill and liver and some selected hematological parameters in the cichlid fish *Pseudetroplus maculatus*.

Materials and methods

Animal:

The cichlid fish, *Pseudetroplus maculatus* (b.wt. 7 ± 1 g and length 6.5 ± 1 cm) were collected from a local fish farm, KKF Nursery, Manjeri, Kerala, India. Fish were acclimatized to laboratory conditions for two weeks prior to the experiments in aquarium (40 L capacity) with dechlorinated water and good lighting system (12 h light: 12 h dark).

Preliminary tests:

The physico-chemical characteristics of the tap water were estimated as per APHA guidelines (1998). Water temperature (28 ± 2 C), oxygen saturation of water (70 to 100 %), and pH (6.5 to 7.5) were continuously monitored throughout the experiment in both control and treatment groups.

Chemicals:

Bisphenol A (4, 4 Isopropylidenediphenol) of 97% purity was obtained from SISCO Research Laboratories Pvt. Ltd., Mumbai, India. All other chemicals were of analytical grade and obtained from local commercial sources.

Treatment:

After two weeks of acclimatization, fishes were kept in different tanks for the experiment maintaining ten animals per group. Since bisphenol A is insoluble in water it was first dissolved in 1% DMSO and therefore used as a solvent (vehicle) control in the experiment. Earlier studies from our laboratory reported the median lethal concentration (96 h LC_{50}) of bisphenol A in *P. maculatus* as 6.48 mg/ L (Asifa and Chitra, 2015). One-tenth of the LC_{50} concentration (648 µg/L) of bisphenol A was selected in the present study as sublethal concentration and it was exposed for short-term (24 h, 72 h, 96 h) and long-term (7 days and 15 days) durations along with negative control and vehicle control groups.

Oxygen consumption of fish was measured by Winkler's method (Welsh and Smith, 1961). Before starting the experiment, initial water sample was collected immediately from

each group without causing any damage to the animal. Then at every 24 h, water sample was collected up to 96 h in short-term groups and at 3 days intervals in long-term groups. The rate of oxygen consumption in each sample was calculated considering net weight of fish by using formula as prescribed by Winkler's method.

At the end of every treatment period, fishes were caught very gently using a small dip net, one at a time with least disturbance. The blood was collected by cardiac puncture in anticoagulant (1% ethylenediamine-tetraacetic acid - EDTA) coated eppendorf tubes. The whole blood was used for the estimation of erythrocyte and leukocyte counts (Rusia and Sood, 1992) in both control and experimental groups.

After collection of blood sample, gill and liver were extirpated at 96 h from both control and treatment groups and stored in 10% buffered formalin for histological examination. After 24 h, the tissues were dehydrated in ascending grades of alcohol and cleared in xylene. Tissues were then embedded for 1 h in molten paraffin wax and impregnated with wax. Sections were cut at 6 µm and stained with haematoxylin and eosin and mounted in DPX. The structural alterations in the tissues of fish of 96 h exposure groups (control and experimental) were observed under light microscope. Photomicrographs were taken using Canon shot camera fitted to the Carl Zeiss Axioscope 2 Plus Trinocular Research Microscope.

Statistical analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA) and the means were compared by Duncan's Multiple Range test using statistical package (SPSS 17.0). Differences were considered to be significant at $p<0.05$ against control groups. Data are presented as mean ± SD for ten animals per group. All biochemical estimations were carried out in duplicate.

Results

Effect of bisphenol A on oxygen consumption

Bisphenol A at 648 µg/L concentration initially increased the rate of oxygen consumption at 24 h and from 48 h it showed a time-dependent reduction (Fig. 1). An increase in rate of oxygen consumption was recorded at 3 day in Bisphenol A treated group and thereafter it decreased till day 15 (Fig. 2).

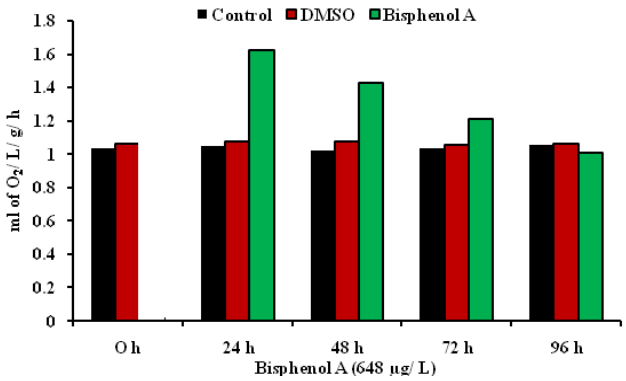

Figure 1: Effect of bisphenol A on oxygen consumption for 96 h in *Pseudetroplus maculatus*

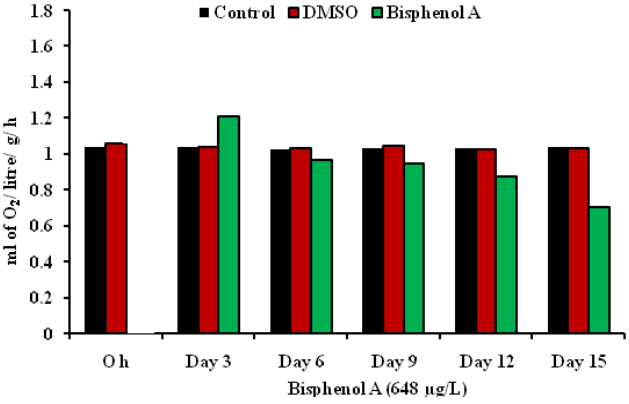

Figure 2: Effect of bisphenol A on oxygen consumption for 15 days in *Pseudetroplus maculatus*

Effect of bisphenol A on haematological parameters

Fish when exposed to sublethal concentration of bisphenol A showed significant ($P<0.05$) decrease in the count of red blood corpuscles after 72 h and 96 h (Fig. 3). A significant decrease in red blood corpuscles count was also recorded in fish exposed to Bisphenol A for 7 and 15 day (Fig. 3).

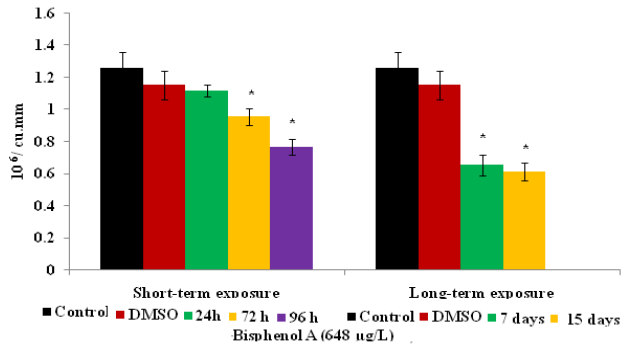

Figure 3: Effect of bisphenol A on erythrocyte count of the fish, *Pseudetroplus maculatus*

A significant increase was recorded in leukocyte count of the fish exposed to short-term (72 h and 96 h) and long-term (7 and 15 day) with Bisphenol A (Fig. 4).

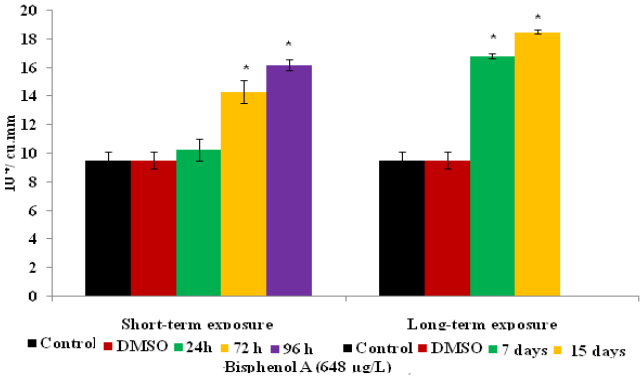

Figure 4: Effect of bisphenol A on leukocyte count of the fish, *Pseudetroplus maculatus*

Effect of bisphenol A on histopathology of tissues

Bisphenol A exposure at sublethal concentration for 96 h showed upliftment of gill epithelium, hyperplasia of gill arches, aneurysm and absence of secondary lamellae in the gill of fish (Fig. 5). Bisphenol A treatment showed disruption in normal architecture of hepatocytes which is revealed by cytoplasmic vacuolization and complete degeneration of cytoplasm after 96 h (Fig. 6).

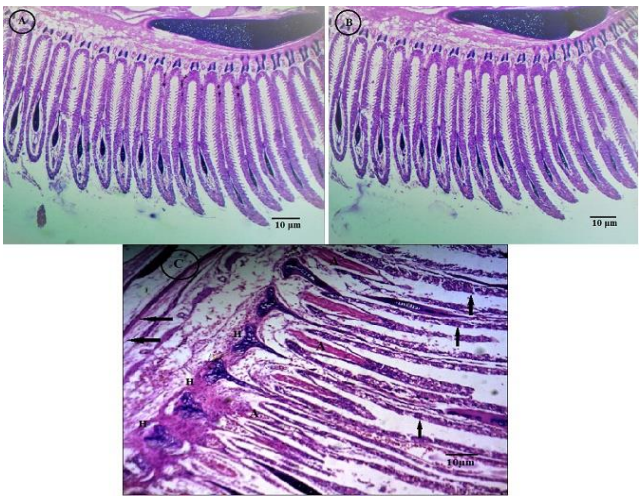

Figure 5: Photomicrograph of gill tissue of the fish, *Pseudetroplus maculatus.* A: Control; B: Vehicle-treated; C: Bisphenol A-treated fish for 96 h showing upliftment of gill epithelium (←), hyperplasia of gill arches (H), aneurysm (A) and absence of secondary lamellae (↑)

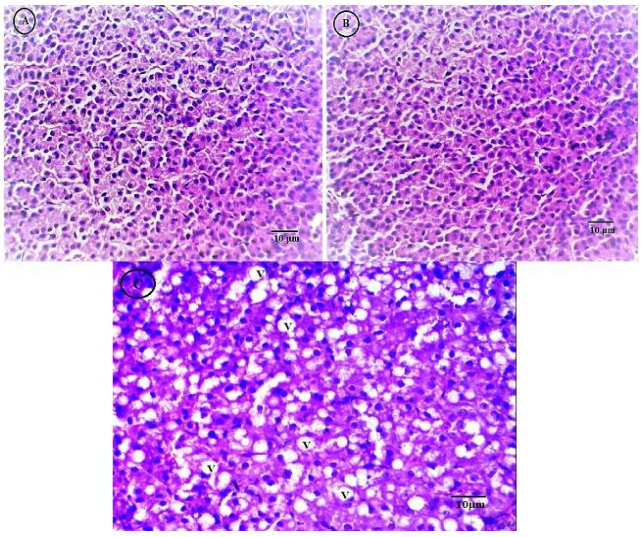

Figure 6: Photomicrograph of liver of the fish, *Pseudetroplus maculatus.* A: Control; B: Vehicle-treated; C: Bisphenol A-treated fish for 96 h showing vacuolization (V) and complete degeneration of cytoplasm

Discussion

In view of the fact that bisphenol A is widely used for the synthesis of plastics, it is frequently been incorporated into a variety of everyday domestic and industrial materials. Thus ponds, rivers and marine waters are contaminated with bisphenol A based products and its effluents from wastewater treatment plants and landfill sites. Bisphenol A can undergo photo-degradation or biodegradation through microbial or plant activities and metabolism by animals in the aquatic environments resulting in relatively less environmental concentration in biota compared to non-biotic environments (Flint *et al*., 2012). But even at very low concentration fishes are highly sensitive to the contaminant. In case of fishes, the major route of chemical exposure is not through the diet but through the gills. Hence, waterborne bisphenol A consistently produces more relevant toxic effects in fish rather than any other animals. Bisphenol A is an estrogen agonist and estrogenic activity is mediated through its binding to estrogen receptors (ERs) in fish (Gibert *et al*., 2011). Exposure to low levels of bisphenol A has been shown to affect rate of body growth, cell differentiation and proliferation, sexual maturation, reproductive function and development, cellular, physiological and genotoxic effects in fish (Sohoni *et al*., 2001; Ramakrishnan and Wayne, 2008; Park and Choi, 2009). It causes induction of oxidative stress in brain and muscle tissues of *Pseudetroplus maculatus* and in the gill of *Oreochromis mossambicus* when exposed at sublethal concentrations (Chitra and Sajitha, 2014; Thulasi *et al*., 2015; Rejitha *et al*., 2016).

The cichlid fish *P. maculatus* is selected in this study to investigate the toxicity potential of bisphenol A as it is an indigenous species of south India and Sri Lanka, inhabiting both freshwater and brackish water habitats. Studies have shown that *P. maculatus* is highly sensitive to various environmental contaminants (Asifa and Chitra, 2016; Sumi and Chitra, 2017). Results obtained in the present study clearly proved that bisphenol A at sublethal concentration decreased the rate of oxygen consumption in time-dependant manner when compared to the control groups. The observed decrease in oxygen consumption by the fish may be due to respiratory distress as an outcome of the impaired oxidative metabolism and/or may be due to the damage in the structural integrity of the cells of respiratory organs induced by the contaminant (Logaswamy and Remia, 2009). Similar results were observed in *Rasbora daniconius* when exposed to dimethoate (Lokhande, 2017) and in *Oreochromis mossambicus* when exposed to quinalphos and silica nanoparticles (Chitra *et al*., 2013; Vidya *et al*., 2016).

In the present study bisphenol A decreased the total red blood cell (RBC) count. This could be due to the decrease in erythropoietic activity or destruction of red blood cells that finally lead to severe anaemic state. The reduction in number of RBCs of stressed fish could also be due to internal bleeding or aggregation of RBCs in damaged gills (Kori-Siakpere *et al*., 2006). Decreased erythocyte count has been noticed in the freshwater fish *Cyprinus carpio* and *Anabas testudineus* after acute exposure to diazinon and ethoxyquin (Svoboda *et al*., 2001; Neethumohan *et al*., 2017). Bisphenol A treatment increased the total leukocyte (WBC) count thus indicating the action of the

contaminant on the immunological defense of the fish. Environmental contaminants like cadmium and glyphosate-based herbicide Roundup Transorb have been shown to induce the WBC count in different fishes like *Pleuronectes flesus* and *Prochilodus lineatus* (Johansson-Sjobeck and Larsson, 1978; Modesto and Martinez, 2010). This may be due to the activation of the immune system in the presence of contaminant, which in turn may be an adaptive response of the organism resulting in a more effective immune defense (Barreto-Medeiros *et al*., 2005).

Gill of control fish is composed of four gill arches on each side of the buccal cavity. Each gill arch is composed of several gill filaments called primary lamellae with two rows of secondary lamellae that lie perpendicular to each filament (Mallatt, 1985). Gill is the site for gaseous exchange and osmoregulation that constitutes over 50 per cent of the total surface area of the animal which makes it more vulnerable to pollutants in the surrounding water. In the present study exposure of sublethal concentration of bisphenol A for 96 h altered normal architecture of gill as revealed by upliftment of gill epithelium, hyperplasia of gill arches, aneurysm, destruction of primary lamellae and absence of secondary lamellae. The lifting of lamellar epithelium could serve as a defensive mechanism because separation of epithelial lamellae increases the distance across waterborne pollutants which are likely to diffuse reaching the bloodstream. Likewise fusion and absence of secondary lamellae reduce the branchial superficial area in contact with the external environment thereby decrease the entry of pollutants into the body of fish (Arellano *et al*., 1999). The damaged gill tissue decreases the diffusion capacity of the gill that leads to decreased rate of oxygen uptake and other respiratory difficulties. Clubbing and degeneration of secondary lamellae, upliftment of lamellar epithelium and hyperplasia of mucous cells were observed in *Catla catla* and *Oreochromis mossambicus* when exposed to sublethal concentrations of bisphenol A (Chitra and Sajitha, 2014; Faheem *et al*., 2016).

Liver of control fish consists of hepatocytes, which are arranged into irregularly shaped lobules separated by hepatopancreas and associated connective tissues. Hepatocytes are parenchymal cells having homogenous cytoplasm with a large central or subcentral spherical nucleus. Liver is site of detoxification and metabolism of xenobiotics, which makes it more vulnerable to environmental contaminants (Eide *et al*., 2014). The present study showed histological alterations in the liver tissue after bisphenol A treatment for 96 h. The degenerative changes observed include vacuolization and necrosis of hepatocytes that finally resulted in complete destruction of hepatic histo-architecture. Occurrence of cytoplasmic vacuolization is related to the altered metabolic functions resulting in accumulation of glycogen in liver cells and is considered as a common response of hepatocytes due to liver injury (Wester and Canton, 1986). Bisphenol A at sublethal concentrations has been shown to cause hepatic tissue damages like vacuolization, necrosis and cavity formation in zebrafish (Cao *et al*., 2010). Similar alterations in liver were also observed in *Oreochromis mossambicus* and *Pseudetropus maculatus* when exposed to di(2-ethylhexyl)phthalate (Revathy and Chitra, 2015) and chlordecone (Asifa and Chitra, 2017).

Conclusion

It is concluded that, acute exposure to bisphenol A at sublethal concentration caused severe toxic effects in the fish which is evident by decreased rate of oxygen consumption, haematological and histological alterations. These alterations indicate the negative health status of fish due to bisphenol A toxicity which indirectly point out the threat to the survivability of the fish species in its natural ecosystem.

Acknowledgement

Financial grant received to carry out this study from Kerala State Council for Science, Technology and Environment (KSCSTE), Thiruvananthapuram, Kerala is gratefully acknowledged.

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