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Biochemical Perturbations and Metabolic Derangements Induced by Benzophenone-3 at Environmentally Relevant Concentration in the Liver of *Danio rerio*

S. Miltonprabu [a*], K. Shagirtha [b] and S. Senthilmurugan [c]

[a] Department of Zoology, University of Madras, Chennai, India.
[b] Department of Biochemistry, St. Joseph's College, Cuddalore, India.
[c] Department of Zoology, Annamalai University, Annamalainagar, India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

UV filters are used daily by millions of people. many wastewater treatments plants are ill-equipped to filter them properly. As a result, UV filters are progressively reaching the environment at an alarming level. Benzophenone-3(BP3) in particular, are toxic to all organisms. Its toxic effects include coral bleaching and interference with metabolic, enzymatic, and reproductive activities. The

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[++] Associate Professor;
[#] Assistant Professor;
**Corresponding author: Email: : miltonprabu@hotmail.com, smprabu73@gmail.com;*

present study aims to assess the toxic impact of environmentally relevant concentration of BP3(44µg/l) on some selected biochemical variables in the liver tissue of Zebra fish on different exposure periods. The exposed fishes were shown to exhibit significant alterations in the carbohydrate, lipid and protein levels and their metabolism and elevated the level of transaminases in the hepatic tissue. The severity of derangement was slowly increased upon increasing exposure period and more significant at the 45th days of exposure. From our results, it may suggested that BP3 exposure at sub chronic periods even at the environmentally relevant concentration might induce intense biochemical alterations in the liver of *D.rerio.*

Keywords: BP3; D.rerio; liver; chronic toxicity; enzymes.

1. INTRODUCTION

"Anthropogenic contamination by UV filters in personal care products, plastics, pesticides, paints, textiles and packaging materials pose a serious threat to the aquatic life due to their over accumulations in the recent times. Organic ultraviolet filters (UVFs) such as benzophenone (oxybenzone), avobenzone and octocrylene are emerging as contaminants of concern in the aquatic environments" [1]. "Among the organic UV-filters, Benzophenone-3 (BP3) is one of the most commonly used organic UV-filter compounds" [2,3]. "Widespread use of BP3 has led to the release of this compound into aquatic environments around the world" [3].

"BP3 belongs to the class of aromatic ketones known as benzophenones. It is a naturally occurring chemical found in various flowering plants" [4]. "BP3 is photostable and lipophilic" [3]. "Under mid-altitude conditions, the half-life of BP3 in surface water was estimated at a couple of weeks in summer time, and over 3 months in winter time suggesting slow degradation in aquatic environments" [5] Benzophenone-3 (Oxybenzone), BP3, is one of the emerging environmental contaminants that is widely utilized in sunscreens as well as in PCPs as it aids in helping to minimize the damaging effects of UV radiations [6]. "BP3 also affects the human health. According to the Center for Disease Control (CDC) fourth national report on human exposure to environmental chemicals, approximately 97% of the people that were tested had oxybenzone in their urine, and additionally, independent scientists worldwide have also reported various concentrations of BP3 in waterways and fish" [7].

"BP3 can accumulate in tissues of organisms because of its lipophilicity and stability. Reports on occurrence of BP3 in biological samples are mainly limited to humans. BP3 and related metabolites have been widely detected in urine, breast milk, serum, cord blood, and placental tissue samples in many countries" [3]. Previously, some researchers reported toxic effects of BP3 that Broniowska et al. [8] reported "BP3 at low concentrations of 10−8 M (0.01 µM) could induce apoptosis in human SH-SY5 neuroblastoma cells". "More recently, it was reported that Zebra fish exposed to BP3 in a range of 32 to 320 µg/L (0.13–1.3 µM) for the first 6 days post fertilization, had reduced thyroid hormone T3" [9]. "In mice, primary neocortical tissue from embryos with prenatal exposure to 50mg/kg BP3 exhibited dysregulated expression of neurogenesis and neurotransmitter-related genes [10], endocrine disruption" [2,11]. and influences reproduction and sex hormone signaling [12]. Further, Tao et al. [13] reported that 24 hrs exposure to BP3 decreased cell proliferation as well as increased apoptosis in the neuronal cells of Zebra fish larvae.

In our previous exploration, we have reported the hepatotoxic nature of BP3 through liver oxidative stress, antioxidant status and histopathological changes in zebra fish [14]. So far, there were no reports existing about the effect of BP3 induced biochemical perturbations and metabolic alterations in the liver of *D. rerio*. Accordingly, the current study was aimed to investigate the effect of environmentally relevant concentration of benzophenone-3 on the biochemical and physiological functions of adult Zebra fish.

2. MATERIALS AND METHODS

2.1 Chemicals

Benzophenone-3 (98% purity) was procured from Sigma Aldrich, USA. Reaming other chemicals and reagents used were of analytical grade and obtained from Merck, Hi media, Mumbai, India.

2.2 Animals

Experimental fish Adult zebra fish (wild-type, AB strain) of both genders (0.5 ± 0.3 g; 3.1 ± 0.4 cm length) were obtained from the Red hills fish farm Chennai, Tamilnadu, India. Fish were acclimatized to laboratory conditions in continuously aerated dechlorinated tap water and maintained under a photo period of 12-h/12-h light-dark cycle. During the acclimatization period, fish were fed twice a day with commercial pellets, and the residues and metabolic wastes were removed daily. 2.3 Stock solution preparation The BP-3 (purity N 98%, Sigma) stock solution (1000 mg/L) was prepared using 100% dimethyl sulfoxide (DMSO) and was stored at −20 °C. The working solution was later prepared by diluting the stock solution immediately before the experiments. The standard solution was added to the experimental vessels with test fish to obtain the environmentally relevant concentration of BP3 (44 µg/L).

2.3 Experimental Setup

The adult zebra fish were exposed to an environmentally relevant concentration of BP-3 (44 µg/L) for 15,30 and 45 days. A group of 100 health fish of the same size were exposed to the selected concentration. Alongside, a control group was also maintained. Three replicates were maintained for each concentration and control groups. The medium and the test solutions were renewed at the end of 24 h for up to 45 days. Feeding was stopped at the different interval (15, 30 and 45 days) and fish were starved 24 h before dissection.

2.4 Sample Preparation

“At the end of 15, 30 and 45 days of exposure, fish (25 nos) were collected from the control and BP-3 exposed groups, washed with distilled water and then blotted dry using tissue paper. The liver tissue was detached from BP-3 treated and control groups. In a Teflon homogenizer, 100 mg of liver tissue and 1.0mL of 0.1 M Tris- HCl buffer (PH 7.5) were added and squeezed. The mixture was centrifuged at 10000 rpm at 4°C for 15 min, and the supernatant was separated and used for biochemical, hepatic markers and antioxidant activity analysis”. [53]

2.5 Biochemical Analysis

Total protein and glycogen content were estimated in the liver by Lowry et al. [15] and Morales et al. [16], respectively. Total cholesterol and triglycerides levels were assessed by Zlatkis et al. [17] and Foster and Dunn [18], respectively. The hepatic markers AST and ALT in liver tissues were estimated by the following method of Reitman and Frankel [19] . Glucose-6-phosphatase in the liver was assayed by the method of Koide and Oda [20]. Hexokinase activity in liver was assayed by using the method of Brandstrup et al. [21]. ACC and FAS activities were assayed by the method of Zimmerman [22] and Cohen [23] respectively.

2.6. Statistics

All the results were presented as mean ± SD of ten fish in each group. The value of $p<0.05$ was considered as statistically significant by one-way analysis of variance (ANOVA) followed by Duncan’s Multiple Range test (IBM SPSS Statistics for Windows, version 15). Dunnett’s post-hoc comparison, were made to test the significance between control and experiment.

3. RESULTS

3.1 Effect of Environmental Relevant Concentration of BP3 on Carbohydrate Metabolism

The Table 1 shows the carbohydrate metabolic markers such as blood glucose, hexokinase and Glucose-6-phosphatase in liver of control and environmental concentration of BP3 exposed Zebra fish. The level of blood glucose was found to be a significant elevation at 30 and 45 days environmental relevant concentration of BP3 exposed Zebra fish then control. However, the blood glucose level was slightly increase at 15 days exposure of BP3 to Zebra fish when compared to control, but declined level of blood glucose non-significant then control.

The activity of hexokinase was significantly decline and glucose-6-phosphatase was significantly increased in liver of Zebra fish exposed to BP3 at environmental relevant concentration at 30 and 45 days then control (Table 1). “At 15 days exposure BP3 at environmental concentration did not bring significant changes in activity of hexokinase and glucose-6-phosphatase in liver of Zebra fish, meanwhile the enzyme activities were slightly altered. The impaired glucose homeostasis observed in the liver of BP3 exposed Zebra fish clearly denoted the adaptive response of the organism to meet out the oxidative stress

induced by BP3. BP3 causes disturbance in the uptake of glucose as well as glucose metabolism. One of the key enzymes in the catabolism of glucose is hexokinase which phosphorylates glucose and converts it into glucose6-phosphate" [24]. "The activity of this enzyme decreased in the liver of BP3 treated fish. Glucose-6-phosphatase catalyzed the final step of glucose production by liver and kidney. An elevated activity of this enzyme was observed in the liver of BP3 treated fish which favors the increased level of glucose to meet out the energy demand upon stress" [24].

The glycogen content was found to be significantly reduced at environmental relevant concentration of UV filter BP3 exposed Zebra fish liver for 30- and 45-days period when compared to control. At the same time, the level of glycogen declined at 15 days BP3 exposed fish, but not-significant than control fish. This favors the glycolytic pathway to produce more glucose to meet out the energy demand during sustained BP3 stress.

3.2 Effect of Environmental Relevant Concentration of BP3 on Lipid Metabolism

In lipid metabolic analysis, the content of triacylglyceride and total cholesterol were noticed in liver of control and BP3 exposed Zebra fish at different intervals (15, 30 and 45 days). The level of triacylglyceride was found to be significantly declined and total cholesterol content increased notably in liver of BP3 treated (Environmental relevant concentration) Zebra fish at 30 and 45 days when compared to control fish. Further, the content of triacylglyceride and total cholesterol were slightly altered at 15 days exposure of BP3 to Zebra fish but changed level was non-significant (Table 1).

3.3 Effect of BP3 on Protein

The Fig.1 displays the content of protein in liver of BP3 exposed and control Zebra fish. The level of protein was significantly declined at environmental relevant concentration of UV filter BP3 treated liver of Zebra fish from 30 and 45 days than control fish, but increased level were non–significant for 15 days exposure than control fish.

3.4 Effect of BP3 on Hepatic Markers

The Fig. 2 displays the content of liver ALT and AST of control and environmental concentration of BP3 exposed Zebra fish at different time intervals (15, 30 and 45 days). The liver ALT and AST levels were found to be significantly ($P > 0.05$) higher in environmentally relevant concentration of BP3 exposed fish than control, at 30 and 45 days. But, the level of AST and ALT were not significant at 15 days BP3 exposed fish when compared to control fish, meanwhile, levels were gradually increased.

Table 1. Effect of UV filter BP3 at environmentally relevant concentration on carbohydrate and lipid metabolism in liver of zebra fish

Groups/ Parameters	Control	BP3 (15 days exposure	BP3 (30 days exposure	BP3 (45 days exposure
Carbohydrate metabolism				
Blood glucose (mmol/L)	7.84 ± 0.60^{a}	8.71 ± 0.72^{ab}	9.96 ± 0.53^{b}	11.81 ± 0.74^{c}
Hexokinase (U/g)	19.46 ± 1.48^{a}	19.03 ± 1.26^{a}	17.21 ± 1.03^{b}	14.47 ± 1.50^{c}
G6Pase (ng/L)	16.43 ± 1.03^{a}	16.18 ± 1.14^{ab}	19.51 ± 1.65^{b}	22.16 ± 1.35^{c}
Glycogen (mg/g)	8.91 ± 0.40^{a}	8.17 ± 0.50^{a}	7.23 ± 0.54^{b}	5.95 ± 0.25^{c}
Lipid metabolism				
TG (mmol/g)	165.0 ± 12.57^{a}	161.32 ± 5.76^{ab}	142.87 ± 8.45^{b}	128.76 ± 5.90^{c}
TC (mmol/g)	20.65 ± 1.57^{a}	20.76 ± 1.04^{a}	23.25 ± 1.19^{b}	25.87 ± 1.90^{c}
ACC (µmoles of acetyl coA con/min/mg)	1.29 ± 0.41^{a}	1.21 ± 0.28^{a}	0.98 ± 0.19^{b}	0.74 ± 0.11^{c}
FAS (nmol/min/mg)	0.79 ± 0.12^{a}	0.71 ± 0.09^{a}	0.49 ± 0.11^{b}	0.31 ± 0.07^{c}
Protein				
Total Protein (mg/g)	9.02 ± 0.14^{a}	8.87 ± 0.10^{b}	6.84 ± 0.05^{c}	5.14 ± 0.04^{d}

Values are expressed as Mean ± SE. Values with different superscript differs significantly at P≤ 0.05(DMRT)

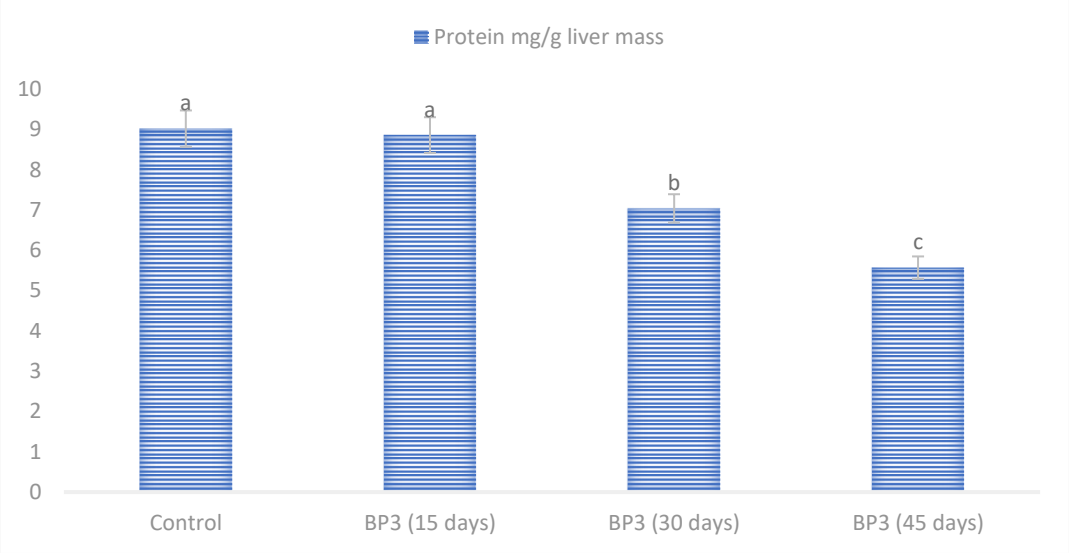

Fig. 1. Effect of BP3 on protein content in the liver of zebra fish

Values are expressed as mean ± SE. The letters (a, b and c) indicate significant differences from the control group Determined by Dunnett's post-hoc comparison, p < 0.05

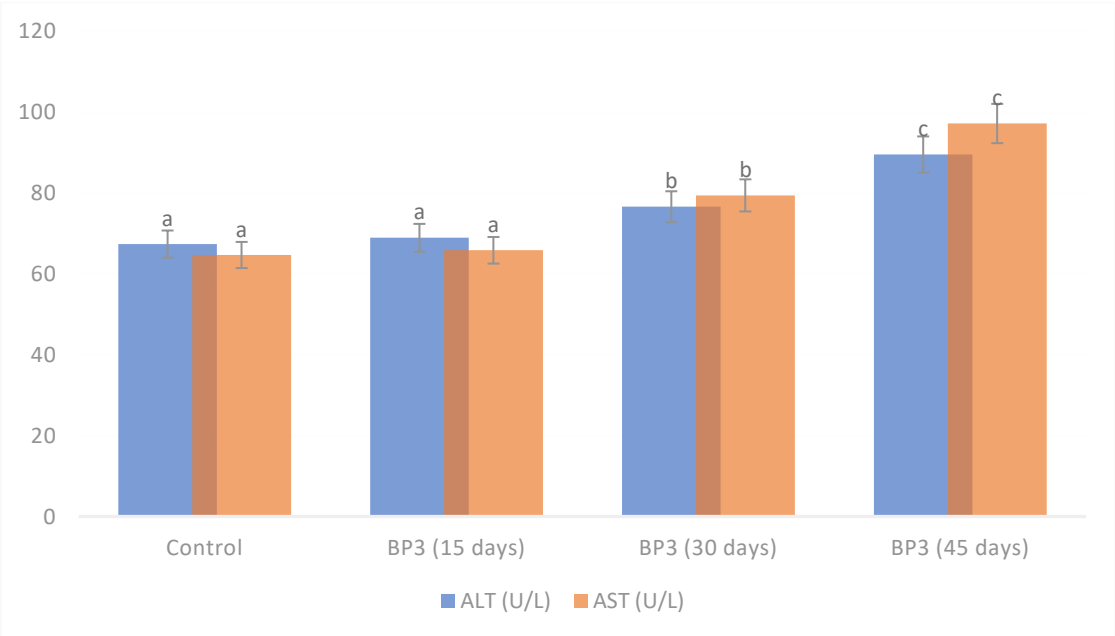

Fig. 2. Effect of BP3 on hepatic markers in liver of zebra fish

The letters (a, b and c) indicate significant differences from the control group (determined by Dunnett's post-hoc comparison, p < 0.05

4. DISCUSSION

Carbohydrates are one of the prime sources of energy for any organism and are found in large amounts in the liver. Lipids play an imperative role and their levels changes as the metabolic activity changes, and also play a vital role in the architectural dynamics of the cell. At higher concentration, pollutants can disrupt the transport mechanism across cell membrane. Changes in carbohydrate and lipid metabolism are to meet the changing energy demands, which can be expected in animals exposed to stress [25,26]. The obtained results of the study primarily indicate that the carbohydrate and lipid metabolism are disturbed when Zebra fish was

exposed to UV filter BP3 at environmental relevant concentration during the experimental period.

The increase in blood glucose concentrations is known as a general secondary response to stress of fish to acute toxic effects and is considered as a reliable indicator of environmental stress [27]. In the present study, the blood glucose level was notably increased in Zebra fish when exposure of UV filter BP3 at environmental concentration (44 µg/L) from 30 and 45 days. The blood glucose was increased in BP3 exposed fish may indicate disrupted carbohydrate metabolism due to enhanced breakdown of liver glycogen, possibly mediated by increase in adrenocorticotrophic and glucagon hormones and/or reduced insulin activity. Earlier, Aly et al. [28] revealed that the pollutants can induce hyperglycemia in fish by altering carbohydrate metabolizing enzymes.

In fish, liver is one of the important organs as it is associated with the coordination of whole-body metabolism in response to nutritional status [29] The liver is the main site of endogenous glucose production, with only a minor contribution from the kidneys through gluconeogenesis and/or through glycogenolysis. Glycolysis and gluconeogenesis are the two primary and complementary events, balancing the glucose load in the body, which is mainly regulated by insulin. Thus, insulin prevents hyperglycemia, in part, by suppressing hepatic gluconeogenesis and glycogenolysis and facilitating hepatic glycogen synthesis [30].

Glycolysis is a process that aids in the breakdown of glucose molecules into pyruvate, accompanying the production of ATP. Glycolysis is an oxygen independent metabolic pathway [31].Hexokinases facilitates phosphorylation of glucose to produce glucose-6-phosphate, which is the first step in the glycolysis pathway. [31] Previously, Kanter et al. [32] stated that “the decreased glycolysis and glucose utilization for energy production, which in turn impairs hexokinase activity”. The present result presented that the hexokinase activity was declined in liver of fish when exposure to BP3 at environmental concentration for 30- and 45-days exposure. This finding indicates that the BP3 may suppress insulin production in beta cells of the Islets of Langerhans of the pancreas, leading to improper glycolysis and glucose utilization.

Glucose-6-phosphatase (G6Pase), an enzyme found mainly in the liver and the kidneys, plays the important role of providing glucose during starvation by generating simple glucose from non-carbohydrate substances and glycogen (Glycogenolysis and Gluconeogenesis) in the liver [33]. The integral endoplasmic reticulum membrane-based enzyme G6PC hydrolyzes its substrate glucose-6-phosphate into glucose. Specifically, G6PC breaks down D-glucose 6-phosphate to D-glucose and orthophosphate. Because G6PC forms with the glucose-6-phosphate transporter (SLC37A4/G6PT), the resulting complex is responsible for glucose production” [34]. In the present investigation, glucose-6-phosphatase was significantly increased in liver of Zebra fish exposed to BP3 at environmental relevant concentration at 30 and 45 days then control. In general, insulin suppresses the glucose production from liver (gluconeogenesis), meanwhile during the starvation/stress stimulate the glucose production from liver. The present study result of increased G6Pase indicates that exposure of BP3 must suppress the synthesis of insulin and insulin sensitivity.

Glycogen is one of the immediate fuel reserves and an important component that can be influenced by stress. The liver is the primary carbohydrate storing site in fish and it plays a very vital role in the homeostasis of blood glucose. [35] The liver maintains a balance between the uptake and storage of glucose in the body [36]. In the present investigation, the glycogen content was decline at environmental relevant concentration of UV filter BP3 exposed Zebra fish liver during the experimental period. The environmental relevant concentration of BP3 pronounced declined level of glycogen, indicating altered metabolism. The depleted levels of glycogen are due to increased demand for these molecules to provide energy for the cellular biochemical process under toxic manifestations made by BP3. The present finding demonstrates that BP3 markedly interfered with glucose metabolism at environmental concentration, which promoted gluconeogenesis and glycogenolysis in the liver, and inhibited glycogen synthesis in the liver and glycolysis. [36]

Lipid metabolism functions in energy metabolism and support various physiological, developmental and reproductive processes [37]. Triglycerides and cholesterol, as main constituents of lipids, play a crucial role in the development of living organisms. Triglycerides present in blood participate in regulation of the bidirectional transference of hepatic fat and blood glucose.

Cholesterol is an essential structural of the cell membrane and lipid raft, as well as a precursor for the biosynthesis of bile acid, steroid hormones and vitamin D [38,39]. Additionally, previous study showed changes in triglycerides and cholesterol induced by toxicants/pollutants could lead to the disorders of lipid metabolism and abnormalities of liver function, which might lead to hyperlipidemia, atherosclerosis and coronary heart disease [37,40]. In the current investigation, the increased level of TC and TG were noticed in BP3 exposed liver of fish. These abnormalities indicate liver damage, which may occur due to oxidative stress induced by BP3. The hepatic steatosis (elevated Tg content) was associated with increased activity of key enzymes involved in liver de novo lipogenesis (ACC and FAS) a clear derangement in lipid homeostasis is observed.

Estimation of total proteins and free amino acid contents of internal organs of tissue forms a crucial factor for toxicological studies [41]. “The proteins are the primary structural and functional polymers in the living systems. They have a broad range of activities, including catalysis of metabolic reactions, transport of vitamins, minerals and oxygen, maintenance of osmotic and ionic regulation, etc. A dynamic equilibrium exists between proteolysis and synthesis, which is mainly responsible for protein turnover and homeostasis in any tissues [42]. Tissue protein content can act as an indicator of xenobiotics-induced stress in aquatic organisms” [43]. The fish exposed to environmental relevant concentrations of BP3 have showed decrease in protein content in liver at all periods. The depletion of protein in tissues after treatment with environmental relevant concentrations of BP3 might be due to reduction in rate of protein synthesis and/or excessive proteolysis during stress condition. Similar results were reported in fishes exposed to other toxicants [40,44]).

The activities of various enzymes are considered to be sensitive biochemical indicators before hazardous effects occur in fish and are important parameters for testing water for the presence of toxicants [45,46]. Transaminases are vital enzymes that are known to be primary players in the mobilization of L-amino acids for gluconeogenesis as well as function as the link between the carbohydrate as well as protein metabolism, under altered physiological and/or pathological conditions [47]. ALT aids to catalyze the transfer of amino group from the alanine to the α-ketoglutarate to generate glutamate as well as pyruvate, while AST catalyze the transfer of the amino group from aspartate to α-ketoglutarate to form glutamate and oxaloacetate [48]. Determination of serum enzymes, such as AST and ALT, is considered a useful biomarker to determine pollution levels during chronic exposure” [45, 49]. Elevation of enzymes AST, ALT and ALP indicates liver damage which may be hepatitis or necrosis of cells [50,49]. In the current study, the serum ALT and AST levels were found to be significantly higher in environmentally relevant concentration of BP3 exposed fish than control fish during the experimental period (15, 30 and 45 days). Thus, the significant increase of these enzymes in the serum seems to indicate liver damage, which may be hepatitis or necrosis of cells of the fish exposed to environmentally relevant concentration of BP3 and similar trends have been previously reported for numerous toxicants exposed to various fishes [51,52]. The present investigation clearly indicated that the treatment with environmental relevant concentration of UV filter BP3 can alter the carbohydrate and lipid metabolism in the liver of Zebra fish.

5. CONCLUSION

Hence, from our present findings, we concluded that constant slow increase in the level of UV filters especially BP3 even at sub lethal concentration potentially increase the risk of inducing toxicity and metabolic alterations in the exposed animals. So we recommend to check and minimize the release of these potential hazardous pollutants into the environment and save our ecosystem.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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