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Effect of benzophenone-3 at the environmentally relevant concentration on the liver of Zebra fish (*Danio rerio* (Hamilton)

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Abstract
The widespread use of Benzophenone-3 (BP-3) has resulted in its release into the ecosystem, and its potential impact on the aquatic ecosystem is of rising concern. Therefore, the current investigation was conducted to analyze the effect of BP-3 at environmentally relevant concentration (44 µg/L in river) in the zebra fish liver through the biochemical markers (protein, glycogen and lipids), hepatic markers, oxidative stress markers and histopathological analysis. The zebra fish was exposure to BP-3 at environmentally relevant concentration for 45 days. During different interval viz. 15, 30 and 45 days, all the above-mentioned markers in the liver and it's histology were analyzed. The biochemical parameters like glycogen, protein triglyceride content were significantly declined with increased total cholesterol level in the zebra fish liver. The activity of hepatic markers like Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST), lipid peroxidation markers viz. thiobarbituric acid reactive substances (TBARS) and Hydrogen peroxide (H_2O_2) were found to be significantly higher meanwhile the activity of antioxidant superoxide dismutase (SOD), catalase (CAT), Glutathione peroxidase (GPx) and Glutathione (GSH) activity were found to be as a significant reduction in the liver of BP-3 treated fish for 30 and 45 days. Furthermore, the liver histology also showed abnormal changes in their morphology when compared to control. At the duration of 15 days, BP-3 exposure slightly altered all the parameters, but the values were non-significant when compared to control fish. From these results, we conclude that the treatment of BP-3 at environmentally relevant concentration can alter the physiology of the zebra fish liver.

Keywords: Benzophenone-3, oxidative stress, lipid peroxidation and hepatic markers

1. Introduction
Benzophenone-3 (BP-3) is a commonly used sunscreen agent, absorbing UVB and UVA radiation. The industrial use of BP-3 has increased over the past decade [1]. BP-3 is utilized as a flavour ingredient, a fragrance enhancer, a perfume fixative, an additive for plastics, coatings, adhesive formulations, as an ultraviolet curing agent in sunglasses and as an agent to prevent the UV light from damaging the scents and colours in perfumes and soaps [2]. Widespread use of BP-3 in personal care products (PCPs) has led to the release of this compound into the aquatic environments around the world [1]. In the aquatic environment, BP-3 ranges from ng to µg/L. The highest concentrations detected are 44 µg/L in the river, 34.3 µg/L in seawater, 10.4 µg/L in wastewater influent, 4.5 µg/L in the swimming pool, 0.45 µg/L in tap water, 0.2 µg/L in the lake, and 0.034 µg/L in groundwater [3]. BP-3 can accumulate in tissues of organisms because of its lipophilicity and stability. Reports on the occurrence of BP-3 in biological samples are mainly limited to humans. BP-3 and related metabolites have been widely detected in urine, breast milk, serum, cord blood, and placental tissue samples in many countries [4].

There were several toxic reports available on the BP-3 such as reduce thyroid hormone T3 [5], decline the reproductive potential [6], endocrine dysfunction [7] and dysregulated expression of neurogenesis and neurotransmitter-related genes [2]. However, Tao *et al*. (2020) [8] have only explored its adverse effects elicited in aquatic organisms at environmentally relevant concentrations. Who reported that at an environmentally relevant concentration of BP-3 can induce neurotoxicity through the decreased axonal growth, reduced cell proliferation, and increased cell apoptosis in the head region of zebra fish larval. There was no report about BP-3 at the environmentally relevant concentration on the physiology of the liver of fish.

The liver is a major organ that is involved in the detoxification and is also the main site of many important metabolic reactions involving carbohydrate, lipids and protein [9]. Fish livers have been a favourite model to study the interactions between environmental factors and the functions and structures of the liver. Therefore, the present investigation aimed to notice the effect of BP-3 at the environmentally relevant concentration on the physiology of the liver of 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, Himedia, Mumbai, India.

2.2 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 photoperiod 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 BP-3 (44 µg/L).

2.4 Experimental design
The adult zebra fish were exposed to an environmentally relevant concentration of BP-3 (44 µg/L) for 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.

2.5 Sample preparation
At the end of 15, 30 and 45 days of exposure periods, 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.

2.6 Biochemical analysis
Total protein and glycogen content were estimated in the liver by Lowry *et al*. (1951) [10] and Morales *et al*. (1973) [11], respectively. Total cholesterol and triglycerides levels were assessed by Zlatkis *et al*. (1953) [12] and Foster and Dunn (1973) [13], respectively. The hepatic markers AST and ALT in liver tissues were estimated by the following method of Reitman and Frankel (1957) [14].

2.7 Lipid peroxidation and antioxidant analysis
The activity of TBARS and H_2O_2 in the liver was estimated by the method of Fraga *et al*. (1988) [15] and Jiang *et al*. (1992) [16], respectively. The total activity of superoxide dismutase (SOD) was determined by the method of Marklund and Marklund (1974) [17]. The activity of catalase (CAT) was determined according to the procedure of Aebi (1984) [18]. The activity of GPx was determined by the method of Rotruck *et al*. (1973) [19]. The non-enzymatic antioxidant GSH in liver tissue was estimated by the method of Ellman (1959) [20].

2.8 Histopathology analysis
The harvested liver tissue samples were fixed for 48 h in 10% buffered formalin and dehydrated by passing through different percentages of ethyl alcohol and water, finally cleaned in xylene and embedded in paraffin. Sections of the tissues (5-6µm thick) were prepared by using a rotary microtone and stained with haematoxylin and eosin dye, which was mounted in a neutral deparaffined xylene medium for microscopical observations.

2.9 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).

3. Results
3.1 Biochemical analysis
The figure 1 and 2 shows the content of glycogen and protein in the liver of BP-3 exposed zebra fish at environmentally relevant concentration and control, respectively. The glycogen and protein levels were found to be a significant reduction at an environmentally relevant concentration of UV filter BP-3 exposed zebra fish liver at 30 and 45 days period when compared to control. At the same time, the level of glycogen declined somewhat at 15 days BP-3 exposed fish, but not-significant then control.

The level of triglyceride was found to be significantly declined, and total cholesterol content increased notably in the liver of BP-3 treated (Environmental relevant concentration) zebra fish at 30 and 45 days when compared to control. Further, the content of triglyceride and total cholesterol were slightly altered at 15 days exposure of BP-3 to zebra fish but changed level was non-significant (Figure 3 & 4).

The liver ALT and AST levels were found to be significantly ($P > 0.05$) higher in the environmentally relevant concentration of BP-3 exposed fish than control at 30 and 45 days. But, the level of AST and ALT were not significant at 15 days BP-3 exposed fish when compared to control, but the levels were found to be gradually increasing (Figure 5).

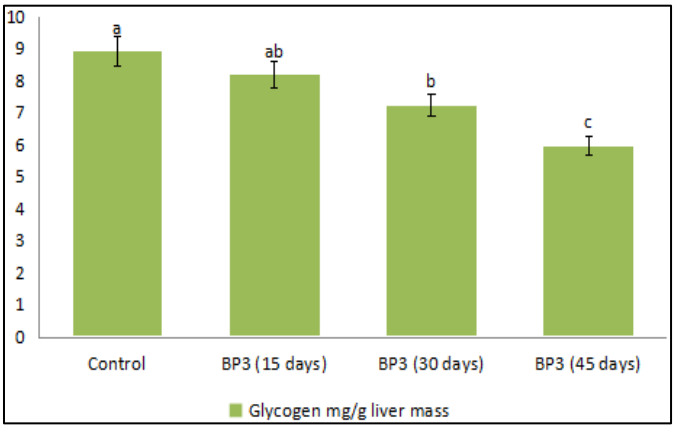

Fig 1: Effect of environmental concentration of BP3 on glycogen in liver of Zebrafish

All the data were expressed as the mean ± S.D. for 10 fish. The results with different superscripts (a, b, c...) for each organ/tissue at different exposure periods (15, 30 and 45) are significantly different at $p<0.05$.

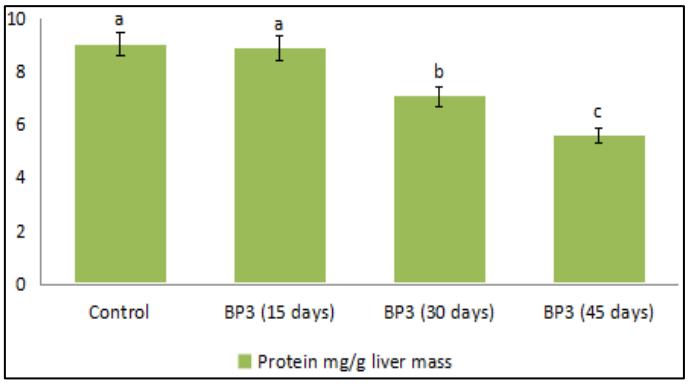

Fig 2: Effect of environmental concentration of BP3 on protein in liver of Zebrafish

All the data were expressed as the mean ± S.D. for 10 fish. The results with different superscripts (a, b, c.) for each organ/tissue at different exposure periods (15, 30 and 45) are significantly different at $p<0.05$.

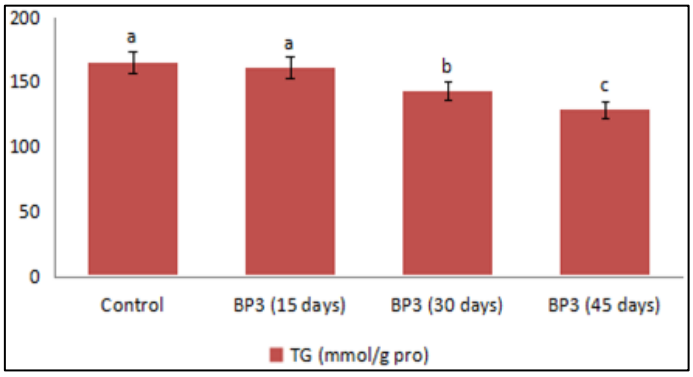

Fig 3: Effect of environmental concentration of BP3 on triglycerides in the liver of Zebrafish

All the data were expressed as the mean ± S.D. for 10 fish. The results with different superscripts (a, b, c.) for each organ/tissue at different exposure periods (15, 30 and 45) are significantly different at $p<0.05$.

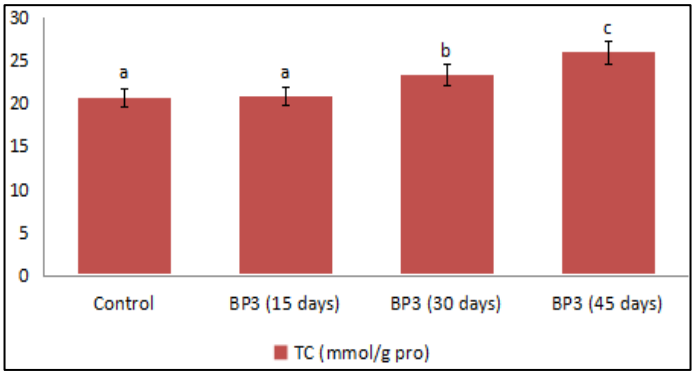

Fig 4: Effect of environmental concentration of BP3 on total cholesterol in the liver of Zebrafish

All the data were expressed as the mean ± S.D. for 10 fish. The results with different superscripts (a, b, c...) for each organ/tissue at different exposure periods (15, 30 and 45) are significantly different at $p<0.05$.

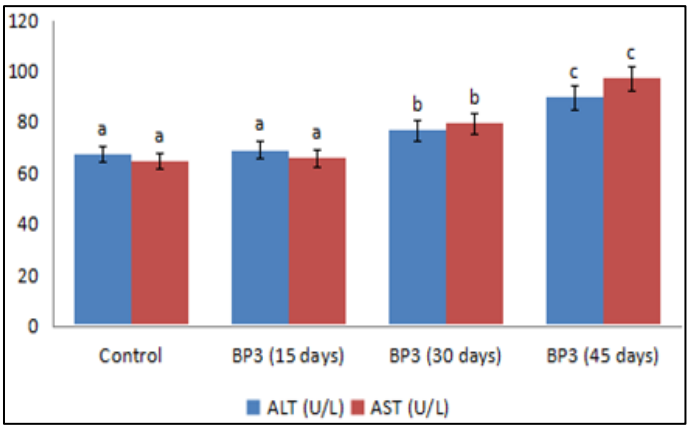

Fig 5: Effect of environmental concentration of BP3 on hepatic markers in the liver of Zebrafish

All the data were expressed as the mean ± S.D. for 10 fish. The results with different superscripts (a, b, c.) for each organ/tissue at different exposure periods (15, 30 and 45) are significantly different at $p<0.05$.

3.2 Effect of BP-3 on lipid peroxidation markers

Figure 6 and 7 represents the activity of TBARS, and H_2O_2 liver of control and BP-3 at environment relevant concentration (44 µg/L) exposed zebra fish. The environmentally relevant concentration exposed zebra fish showed significantly increased activities of TBARS and H_2O_2 in the liver, as compared to normal fish. A significant ($p<0.05$) progressively increased activity of TBARS and H_2O_2 in the tissues were observed from 30, and 45 days of BP-3 exposure, whereas not significant at 15 days.

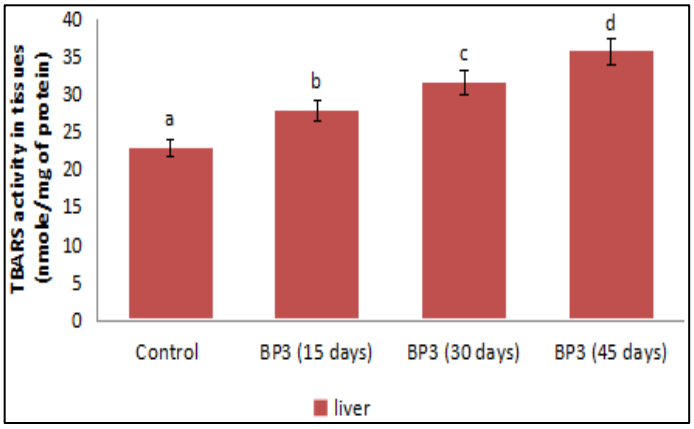

Fig 6: Effect of UV filter BP3 on lipid peroxidation marker TBARS in the liver of Zebrafish

All the data presented were expressed as the mean ± S.D n=10 fishes. The values with different superscripts, i.e., a, b, c, etc., for each organ/tissue at three different exposure periods (15, 30 and 45 days) are significantly different at $p<0.05$.

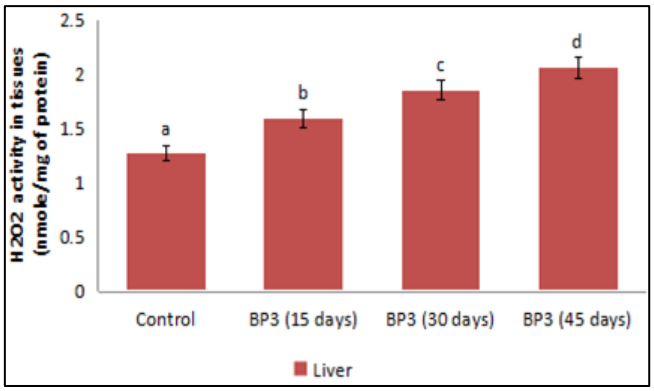

Fig 7: Effect of UV filter BP3 on lipid peroxidation marker hydrogen peroxide in the liver of Zebrafish

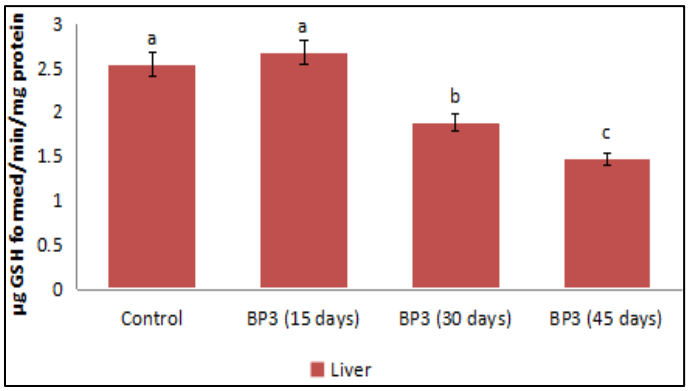

Fig 8: Effect of UV filter BP3 on non-enzymatic antioxidant GSH in the liver of Zebrafish

All the data presented were expressed as the mean ± S.D n=10 fishes. The values with different superscripts, i.e., a, b, c, etc., for each organ/tissue at three different exposure periods (15, 30 and 45 days) are significantly different at $p<0.05$.

3.3 Effect of BP-3 on enzymatic antioxidant

The results of antioxidant activities of SOD, CAT and GPx in the liver of control and BP-3 exposed fish was tabulated in table 1. The antioxidant of SOD, CAT and GPx activities were notably declined in the environmentally relevant concentration of BP-3 exposed zebra fish as compared with control. A significant declined activity of SOD, CAT and GPx were found to be at 30, and 45 days BP-3 treated fish liver when compared to control, meanwhile, the activity was slightly increased from 15 days exposed fish then control.

Table 1: Effect of BP3 on enzymatic antioxidant in the liver of Zebrafish

Groups / parameters	Sod	Cat	GPX
Control	37.56 ± 2.86^{a}	31.38 ± 2.39^{a}	13.89 ± 0.56^{a}
BP3 (15 days)	38.87 ± 1.78^{a}	32.31 ± 1.65^{a}	14.65 ± 0.98^{a}
BP3 (30 days)	32.54 ± 1.95^{b}	28.39 ± 2.54^{b}	10.87 ± 0.84^{b}
BP3 (45 days)	29.61 ± 2.48^{c}	25.51 ± 1.87^{c}	8.94 ± 0.89^{c}

SOD in tissues were expressed as 50% inhibition of nitroblue tetrazolium reduced in 1minute/mg protein; CAT in tissues were expressed as µmoles of H_2O_2 consumed/minute/mg protein. GPx in tissues were expressed as µg of GSH consumed /minute/mg protein. All the data presented were expressed as the mean ± S.D n=10 fishes. The values with different superscripts, i.e., a, b, c, etc., for each organ/tissue at three different exposure Periods (15, 30 and 45 days) are significantly different at $p<0.05$.

3.4 Effect of BP-3 on non-enzymatic antioxidant

Figure 8 represents the level of GSH in the liver of control, and BP-3 exposed zebra fish. The activity of GSH was slightly increased from 15 days exposure of environmentally relevant concentration of BP-3 when compared to control fish liver. Whereas, the level of GSH was significantly declined from 30 and 45 days exposure to BP-3 fish tissues when compared to control.

All the data presented were expressed as the mean ± S.D n=10 fishes. The values with different superscripts, i.e., a, b, c, etc., for each organ/tissue at three different exposure periods (15, 30 and 45 days) are significantly different at $p<0.05$.

3.5 Liver histopathology

In the control fish liver, no histopathological changes were observed. The hepatic parenchyma was arranged with sinusoid-limited hepatocyte plaques. Each of the plates were formed by polarized hepatocytes with a sinusoidal and biliary face. The hepatocytes displayed polygonal shapes along with granular cytoplasm with round nuclei (Figure 9 A). Among the treated groups, the 15 days exposure group (BP-3-15 days) presented some histological modifications like hepatocyte as well as nucleus hypertrophy, along with hyperemia into capillary (Fig. 9 B). The fish from 30 and 45 days exposure groups, i.e., BP-3- 30 days and BP-3- 45 days groups, respectively (Figure 9 C & 9 D), showed highly disorganized liver parenchyma along with sever hepatocyte as well as nucleus hypertrophy and vacuolization in the tissues.

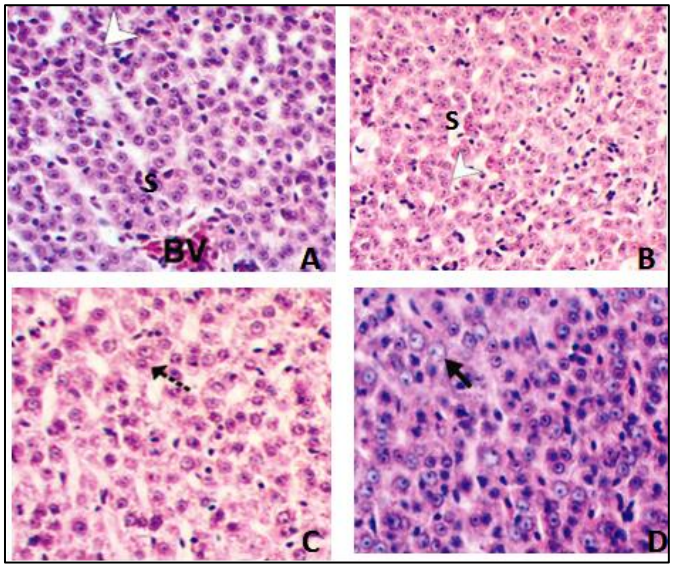

Fig 9: Effect of BP3 at environmentally relevant concentration on histology of liver of Zebrafish

Control group (A), the groups treated with benzophenone-3 for 15 days (B), 30 days (C) and 45 days (D) stained with Hematoxylin and Eosin (H&E, 200x). White (healthy hepatocyte) and black (cytoplasmic vacuolation) arrowheads; BV-blood vessel; S-sinusoid capillary; black arrow nuclear hypertrophy; dotted arrow cytoplasmic degeneration.

4. Discussion

The liver is a major organ, and it is associated with the coordination of whole-body metabolism in response to nutritional status [21]. The obtained results of the study primarily indicate that the zebra fish liver disturbed when exposed to UV filter BP-3 at environmentally relevant concentration during the experimental period.

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 [22], and it plays a very vital role in the homeostasis of blood glucose. The liver maintains a balance between the uptake and storage of glucose in the body [23]. In the present investigation, during the experimental period, the glycogen content was found to decline at an environmentally relevant concentration of UV filter BP-3 exposed zebra fish liver. The environmental relevant concentration of BP-3 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 BP-3. The present finding demonstrates that BP-3 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.

Tissue protein content can act as an indicator of xenobiotic-induced stress in aquatic organisms [24]. The fish exposed to environmental relevant concentrations of BP-3 have showed decrease in protein content in liver at all periods. The depletion of protein in tissues after treatment with environmental relevant concentrations of BP-3 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 [25, 26].

Triglycerides and cholesterol, as main constituents of lipids, play a crucial role in the development of living organisms. Triglyceride present in blood participates 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 [27, 28]. 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 [26]. In the current investigation, the increased level of TC and declined level of TG were noticed in BP-3 exposed liver of fish. These abnormalities indicating liver damage, which may occurs due to oxidative stress induced by BP-3

Determination of serum enzymes, such as AST and ALT, is considered a useful biomarker to determine pollution levels during chronic exposure [29, 30]. Elevation of enzymes AST, ALT and ALP indicates liver damage which may be hepatitis or necrosis of cells [30, 31]. In the current study, the serum ALT and AST levels were found to be significantly higher in environmentally relevant concentration of BP-3 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 BP-3 and similar trends have been previously reported for numerous toxicants exposed to various fishes [32, 33].

Lipid peroxidation (LPO) is a well-established and widely studied cellular mechanism associated with cell injury in animals, and is also used as an oxidative stress indicator for cells as well as tissues. The results in obtained show that in BP-3, at environmentally relevant concentration, exposed fish displayed an elevation in the markers of oxidative stress, namely lipid peroxidation as TBARS and hydrogen peroxide (H_2O_2). TBARS method is generally utilized to measure the Malondialdehyde (MDA) in biological samples [34]. In the current study, TBARS level was significantly increased ($p<0.05$) in various tissues of environmentally relevant concentration of UV filter BP-3 exposed fish than control fish. The increase may be due to the ROS production in the tissues of BP-3-exposed fish. The generation of H_2O_2 is a normal attribute of cellular mechanism, the increased production of H_2O_2 may lead to oxidative stress when the cellular antioxidant defence system is over whelmed or compromised. In the present investigation, the level of H_2O_2 was found to be higher in tissues of UV filter BP-3 exposed fish when compared to control. This result indicates that, the accumulation of BP-3 in tissues may induce ROS production, and affects the CAT activity, which catalyses and neutralizes the H_2O_2 derived from oxidative stress. A previous study reported, for the first time, that BP-3 induce lipid peroxidation in fish. However, previously Huang *et al*. (2020) [35] presented that UV filters such benzophenone-4 (BP-4), 4-aminobenzoic acid (PABA), and 2-phenylbenzimidazole-5-sulfonic acid (PBSA) increases the LPO marker Malondialdehyde in zebra fish liver, which significantly changed during the whole exposure period.

The enzymatic antioxidant system, i.e., SOD, CAT and GPx, plays an important role by coordinating and preventing oxidative damage by ROS in the biological system [36]. SOD catalyzes the binding of ROS with water to generate H2O2 and it is the first enzymatic defense against the superoxide anion [37]. CAT is responsible for the breakdown of H2O2 to water and oxygen, protecting the cell from the damaging action of H2O2 and the hydroxyl radical [38]. GPx catalyzes the reaction of hydro peroxides with reduced GSH to form glutathione disulfide and the reduction product of the hydro peroxide [39]. In the current study, the activities of SOD, CAT and GPx were found to be significantly declined in the tissues of UV filter BP-3 exposed fish at 30 and 45 days exposure periods, whereas the activity of these enzymes were slightly increased at 15 days exposure when compared to the control group. These enzyme activities were found to be declined with extended duration of BP-3 exposure, which may be due to the inhibition of these enzymes by increased cellular H_2O_2 levels. This findings supported by Huang *et al*. (2020) [35], reported that the activity of SOD and CAT in the liver of zebra fish was inhibited by UV filters BP4, PABA and PBSA exposure.

GSH is the major non-protein thiol and plays a pivotal role in cell viability protecting cells against lipid peroxidation either alone or in conjugation with other proteins [40]. In previous studies, they

have reported that the exposure to organic pollutants may result in either increase or decrease of GSH levels in the test organisms [41, 42]. Our results showed that the content of GSH was found to be low in the tissues of zebra fish exposed to environmentally relevant concentration UV filter BP-3. This reduction in the GSH level may be due to the accumulation of BP-3 that lead to the increased utilization or depletion (oxidation to GSSG) of GSH for the detoxify of BP-3 and increased production ROS in the tissues due to BP-3 accumulation. The present result was supported by Gao *et al*. (2013) [43], who reported the content of GSH was declined in *Tetrahymena thermophila* to expose BP-3. Among the vital organs, liver plays a very vital role in the accumulation as well as detoxification of numerous contaminants. Moreover, the increase in exposure concentration of toxicants is directly proportional to the degree of the liver tissue architectural damage [44]. The result of this study showed disorganized liver parenchyma along with sever hepatocyte as well as nucleus hypertrophy and vacuolization in the tissues in BP-3 treated groups compared with the control group. The abnormal changes of hepatocytes may be a result of the production of ROS by BP-3, which cause damage to the hepatocytes and in turn, disrupts the normal functioning and architecture of the liver.

5. Conclusion

The current results presented that at environmentally relevant concentration of BP-3 (44 µg/L) can alters protein, glycogen, cholesterol levels and the activity of enzymes such as, enzymatic antioxidants (SOD, CAT and GPx), non-enzymatic antioxidant GSH as well as hisology in the liver of zebra fish. Thus, it is evident that the long-term exposure of BP-3, at environmentally relevant concentration, imposes a threat on the normal development of economically important fish and in turn may affect the economic as well as health status of the aquaculture industries and the consumers of fishes, respectively.

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