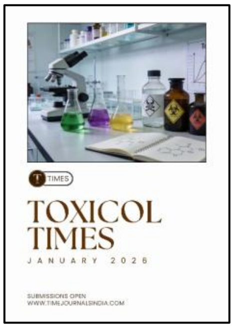

Assessment of 4,4'-Dihydroxy Benzophenone Induced Developmental, Cardiac and Neurotoxic Effects on Zebrafish (*Danio rerio*) Embryo-Larvae

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ABSTRACT

The increasing use of organic UV filters, such as benzophenone (BP-3) and its derivatives have raised concern regarding their ecotoxicological effects on the aquatic environment. 4,4'-Dihydroxybenzophenone (DHBP), a derivative of benzophenone is used in many cosmetics products to protect the skin against the harmful effects of UV radiation. DHBP has been detected in the environment and become an emerging contaminant of concern. However, no work was done on DHBP in previous years. The aim of the present study was to examine the embryo-larval toxicity attributed to DHBP, utilizing zebrafish (*Danio rerio*) as a model after exposure to environmentally relevant concentrations of this compound. Zebrafish embryos were exposed to DHBP at concentrations of 200, 400, 800 µg/L at different time intervals i.e. 24, 48, 72 and 96hours post fertilization (hpf) to examine developmental toxicity, cardiac toxicity, neurotoxicity and effects on growth parameters. DHBP significantly elevated the mortality rate and depleted the hatchability rate in a concentration and time dependent manner at different exposed groups when compared to control group. Heart rate was found to be significantly increased in DHBP exposed groups when compared to control. The neurotoxicity marker i.e. spontaneous tail coiling movement was found to be significantly decreased in DHBP exposed larvae compared to control. The growth parameters were also affected after exposure such as yolk sac area was significantly reduced in 200 µg/L exposed group of DHBP when compared with control. Data demonstrated that, in general, environmentally relevant concentrations of DHBP did not significantly induce changes in SV-BA distance, pericardial area, body length and eye size in DHBP exposed groups when compared to control. Altogether, the data indicates that a potential ecotoxicological impact on aquatic environment exists.

KEYWORDS: UV Filters; Hatchability Rate, Morphological Deformities, Growth Parameters, Heart Rate, Spontaneous Tail Coiling Movement

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1. INTRODUCTION

Organic UV filters are used in a wide range of products and plastics to protect human skin and materials from the harmful effects of UV radiation (Fent *et al*., 2010; Waldman *et al*., 2019). In the 1950s, benzophenones (BPs) and their derivatives were introduced into sunscreens (Heurung *et al*., 2014). Oxybenzone, a derivative of benzophenone, is a common organic UV filter used in sunscreens, cosmetics and personal care products (Giokas *et al*., 2005). These compounds efficiently absorb and dissipate UV radiation, preventing photo-induced degradation in cosmetics and industrial products (Fent *et al*., 2010). As UV filters are emerging pollutants, a major effort must be made to develop UV filter risk assessment and to understand their toxicity in the aquatic environment. Oxybenzone is one of the most frequently detected UV filters in surface waters and wastewater. Since 2005, it has been listed as an emerging contaminant owing to its worldwide occurrence (Richardson *et al*., 2018).

Similarly, 4,4'-Dihydroxybenzophenone is also an organic compound commonly used as a UV absorber in plastics, coatings, and cosmetics to prevent photodegradation (Parker *et al*., 2002). It has two hydroxyl groups attached to a benzophenone structure, enhancing its ability to absorb UV light. BP-3 is another broad-spectrum UV absorber which can effectively absorb UV light at 290-440nm (Heurung *et al*., 2014). It has good stability. Environmental concentrations of BP-3 in natural water range between 0.7 and 7.8 µg/L (Balmer *et al*., 2005). In the USA, an incredibly high concentration of 1.395 mg/L was detected along Trunk Bay in the Virgin Islands (Downs *et al*., 2016; Balmer *et al*., 2005). The widespread use of benzophenone type filter resulted in their continuous release into the aquatic systems. The direct release of benzophenone is primarily due to human activities (e.g. swimming and bathing), industrial wastewater discharges and laundry activities (Bluthgen *et al*., 2012; Du *et al*., 2017; Zhang *et al*., 2017). However, indirect release from wastewater treatment plant effluents remains are the main sources of benzophenone and its derivatives in the environment. Once in natural aquatic systems, benzophenone can accumulate and negatively impact the ecosystem (Sun *et al*., 2021). Human exposure to benzophenone occurs mainly through dermal absorption, ingestion and inhalation, particularly from personal care products and food packaging (Krause *et al*., 2012).

Benzophenone was detected in various biological samples such as, in human urine samples (upto 1.1 mg/L), human amniotic fluid (upto 15.7 µg/L) in a Denmark study (Krause *et al*., 2018) and in placenta (upto 9.8 ngg^{-1}) (Mao *et al*., 2022). Several benzophenone derivatives exhibit hormone disrupting properties, potentially affecting reproductive health and thyroid function (Schlumpf *et al*., 2010). These findings indicate that humans are readily exposed to benzophenone, which may lead to health issues. Several studies have identified benzophenone exposure to developmental toxicity, hormonal disturbances and carcinogenic potential in both humans and wildlife (Heurung *et al*., 2014). For instance, Hawaii banned oxybenzone in 2018 due to its harmful effects on coral reefs and the European union (EU) has regulated BP-3 concentrations in cosmetics due to its endocrine disrupting effects (Down *et al*., 2016).

Zebrafish (*Danio rerio*) is considered as an excellent model organism in various biomedical fields, including developmental toxicology (Ota *et al*., 2014). Because larvae and embryo of zebrafish are transparent and can be easily visible under the microscope. Zebrafish undergo rapid development, facilitating the evaluation of various toxicological endpoints within a few days post fertilization. Zebrafish larvae and embryo are used to test the toxicity due to their high sensitivity to environment pollutants at low level (Felix *et al*., 2013).

There has been limited prior research focused specifically on 4,4'-Dihydroxybenzophenone (DHBP), particularly regarding its toxicological impacts on aquatic organisms. While DHBP is widely used as a UV stabilizer in various consumer products, including plastics and cosmetics, its persistence in the environment and potential for long-term biological effects remain poorly understood. Most existing studies have focused on more common UV filters such as oxybenzone or benzophenone-3, leaving a significant gap in the scientific literature for DHBP. This lack of comprehensive data poses challenges for regulatory assessment and environmental safety evaluations.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

The chemical 4,4'-dihydroxybenzophenone (CAS: 611-99-4, Purity: 99%) was purchased from Sigma-Aldrich and stock solution was prepared in dimethyl sulfoxide (DMSO, > 99%, Fisher Scientific). The final concentrations were made in E3 media (5 mM NaCl,0.33 mM $CaCl_2$, 0.17 mM KCl and 0.33mM $MgCl_2$; pH 7.4).

2.2 Zebrafish housing and embryo selection

Wild-type AB strain zebrafish (*Danio rerio*, aged 5-6 months) were obtained from animal house, Guru Nanak Dev University, Amritsar. They were acclimatized for at least 15 days in a glass aquarium containing 40 liters of dechlorinated water, is well aerated prior to experiment. The following environmental conditions were maintained during acclimatization; temperature 26±2°C, pH ranging from 7.2 to 7.5, water conductivity between 479 and 520µS/cm and a light/dark cycle of 14h/10h. Throughout the acclimatization, the fish were fed twice daily with micro-pellets. After acclimatization, fish were separated (males and females) and fed with dried egg feed, dried crushed brine shrimp and pellets thrice a day. Random mating was done between sexually mature male and female in ratio 3:2 in a breeding tank to obtain zebrafish eggs. According to Kimmel *et al*. (1995) fertilized and unfertilized eggs were separated out with the help of stereomicroscope. The embryos at 2-3 hours post fertilization were selected for further experimental exposure. According to animal welfare act and European legislative directive 2010/63/EU, no animal test authorization is needed for zebrafish embryos and larvae until 120hpf.

2.3 Exposure experiment

On the basis of literature and concentrations of benzophenone and its derivatives detected in environment (air, water, soil etc.) (Down *et al*., 2016; Zhang *et al*., 2017; Sun *et al*., 2021). Three concentrations were selected for present study i.e. 200, 400, 800 µg/L DHBP. For each concentration, 50 embryos were exposed per 50 mL of E3 media. For exposure experiment, there were five groups i.e. control (only E3 media), solvent control (DMSO), 200 µg/L, 400 µg/L and 800 µg/L DHBP. The exposure duration was up to 96 h. The test concentrations were made in freshly prepared E3 media. The experiment was performed in triplicates. The test chemicals were renewed after every 24 h till the end of experiment. The exposure was done in glass petri dishes and kept in an incubator at temperature 26±2°C and under 12h:12h light/dark cycle. After 24, 48, 72 and 96h of exposure, dead embryos were removed immediately to avoid the contamination.

2.4 Developmental toxicity observations

During exposure period of DHBP, embryos were examined daily (24, 48, 72 and 96h of exposure) using the EVOS XL Core Invitrogen microscope imaging system (Thermo Fisher Scientific). For developmental toxicity assessment, mortality rate, hatchability rate and morphological deformities were observed. Mortality rate was observed at 24, 48, 72 and 96h of exposure. The zebrafish embryo-larvae that appeared opaque, showed no movement or had no heartbeat were considered dead (Ali *et al*., 2011) and were removed from the petri dishes to prevent contamination. Hatchability rate was observed after 72 and 96h of exposure whereas morphological deformities were observed after 96h of exposure.

2.5 Growth parameters observations

After 96 h of DHBP exposure, 10 larvae were randomly selected from each treatment group and immobilized using 3.5% methylcellulose. Images were captured using the EVOS XL Core Invitrogen microscope imaging system (Thermo Fisher Scientific). Morphometric parameters, including total body length (head to tail), eye size and yolk sac area, were measured from the captured images using ImageJ 1.54d software (National Institutes of Health, Bethesda, MA, USA).

2.6 Cardiac toxicity assessment

For the assessment of cardiac toxicity, 10 larvae were randomly selected from each treatment group. Cardiac parameters including heart rate, sinus venosus–bulbus arteriosus (SV–BA) distance, and pericardial area were evaluated. Videography was performed using the EVOS XL Core Invitrogen microscope imaging system (Thermo Fisher Scientific) to determine the heart rate of zebrafish larvae after 72 and 96 h of exposure. Images captured after 96 h of exposure were further analyzed to measure SV–BA distance and pericardial area using ImageJ 1.54d software (National Institutes of Health, Bethesda, MA, USA).

2.7 Neurobehavioral toxicity assay

Spontaneous tail coiling movement was used to evaluate the neurobehavioral toxicity of the exposed chemical. Videos of randomly selected zebrafish embryos were recorded for 30 seconds at 24 h of exposure using the EVOS XL Core Invitrogen microscope imaging system (Thermo Fisher Scientific). The number of tail coiling movements was then counted to assess neurobehavioral alterations.

2.8 Statistical analysis

All statistical analysis was done using SPSS version 21. Data are expressed in mean ± standard error (SE). One-way analysis of variance (ANOVA) and Tukey's post hoc test were used to assess the statistically significant differences between different groups. Before analysis, data were tested for normality (Shapiro–Wilk test) and homogeneity of variance (Levene's test).

3. RESULTS

3.1 Developmental toxicity and morphological abnormalities

The mortality rate was found to be significantly ($p \leq 0.01$) elevated in a time and concentration dependent manner in DHBP exposed groups when compared to the control group **(Fig.1A)**. After 24 h of exposure, the mortality rate was observed to be 17.33%, 27.33% and 36.00% whereas after 96 h of exposure, the mortality rate was observed to be 21.33%, 40.67% and 47.33% in 200 µg/L, 400 µg/L and 800 µg/L exposed groups, respectively. The hatching rate in DHBP exposed groups was found to be significantly ($p \leq 0.01$) depleted in a concentration dependent manner when compared to control group **(Fig.1B)**. The hatching rate after 72 h exposure was found to be 73.33%, 48.67% and 49.33% in 200 µg/L, 400 µg/L and 800 µg/L exposed groups, respectively whereas 71.33% hatchability rate was reported in control group. After 96 h of exposure, the hatchability rate was found to be 78.67% (in 200 µg/L DHBP), 59.33% (in 400 µg/L DHBP) and 52.67% (in 800 µg/L DHBP) whereas in control group, 90% of hatching rate was observed.

Zebrafish embryos-larvae exposed to 200, 400 and 800 µg/L concentrations of DHBP showed tail deformities including bent spine and crooked tail after 96h exposure. Other deformities, such as pericardial edema and yolk sac edema were also observed **(Fig. 2)**.

3.2 Growth parameters

The body length, eye size and yolk sac area in zebrafish larvae exposed to different concentrations of DHBP are as shown in **Fig. 3**. After 96h of exposure, total body length of zebrafish larvae was significantly ($p \leq 0.01$) depleted at 400 µg/L (3.24±0.08) and 800 µg/L (2.98±0.04) but there was no significant difference observed at 200 µg/L DHBP exposed group when compared to control group (3.48±0.02). No significant difference was observed in eye size after 96 h of DHBP exposure. The yolk sac area was only found to be significantly ($p \leq 0.05$) reduced by 21.73% in 200 µg/L DHBP exposed group when compared to control group. No significant difference was observed in yolk sac area in 400 and 800 µg/L DHBP exposed groups.

3.3 Cardiac toxicity

The heart rate was observed to be significantly ($p \leq 0.01$) increased in a concentration dependent manner in DHBP exposed groups (200, 400, 800 µg/L) when compared to control group **(Fig.4A)**. After 72 h of exposure, heart rate was observed to be increased up to 61.97±0.09 (in 200 µg/L), 64.83±0.24 (in 400 µg/L) and 64.83±0.24 (in 800 µg/L) when compared to control group (55.10±1.68). Similarly, the heart rate was found to be increased in DHBP exposed groups as 67.27±0.20 (in 200 µg/L), 67.53±0.29 (in 400 µg/L) and 68.00±0.15 (in 800 µg/L) when compared to control group (57.53±0.20). But no significant difference was observed in SV-BA distance and pericardial area in larvae exposed to the different concentrations of DHBP **(Fig 4B, C)**.

3.4 Neurotoxicity

The first motor activity generated by developing neurons network in zebrafish embryos is represented by spontaneous tail coiling movement. This parameter is used to determine the neurotoxicity potential of any compound. Spontaneous tail coiling movement was found to be significantly ($p \leq 0.01$) reduced by 18.71% (in 400 µg/L) and 30.40% (in 800 µg/L) but no significant difference was observed in 200 µg/L DHBP exposed group when compared to control group **(Fig. 4D)**.

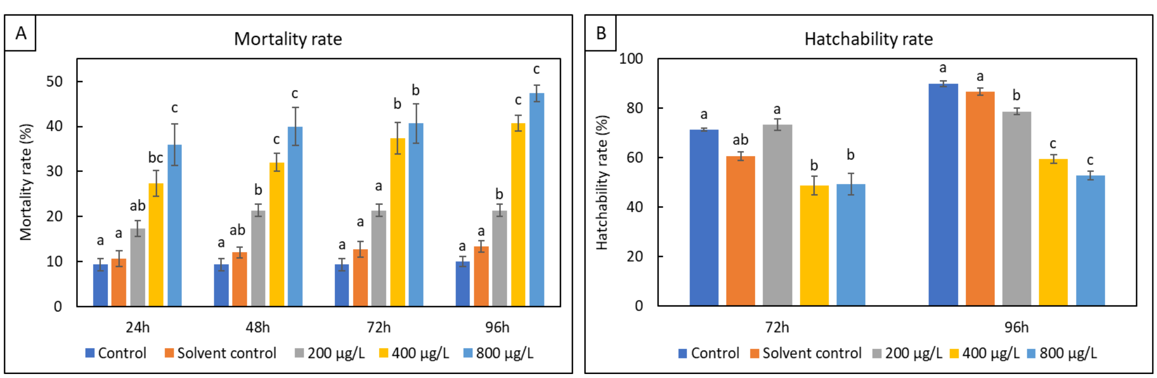

Fig. 1: Mortality and hatchability rate (Mean±SE) in Zebrafish embryos-larvae exposed to DHBP at different concentrations and durations. (A) Mortality rate (B) Hatchability rate. a, b, c signifies the effect of different concentrations at different hours of exposure (n=50).

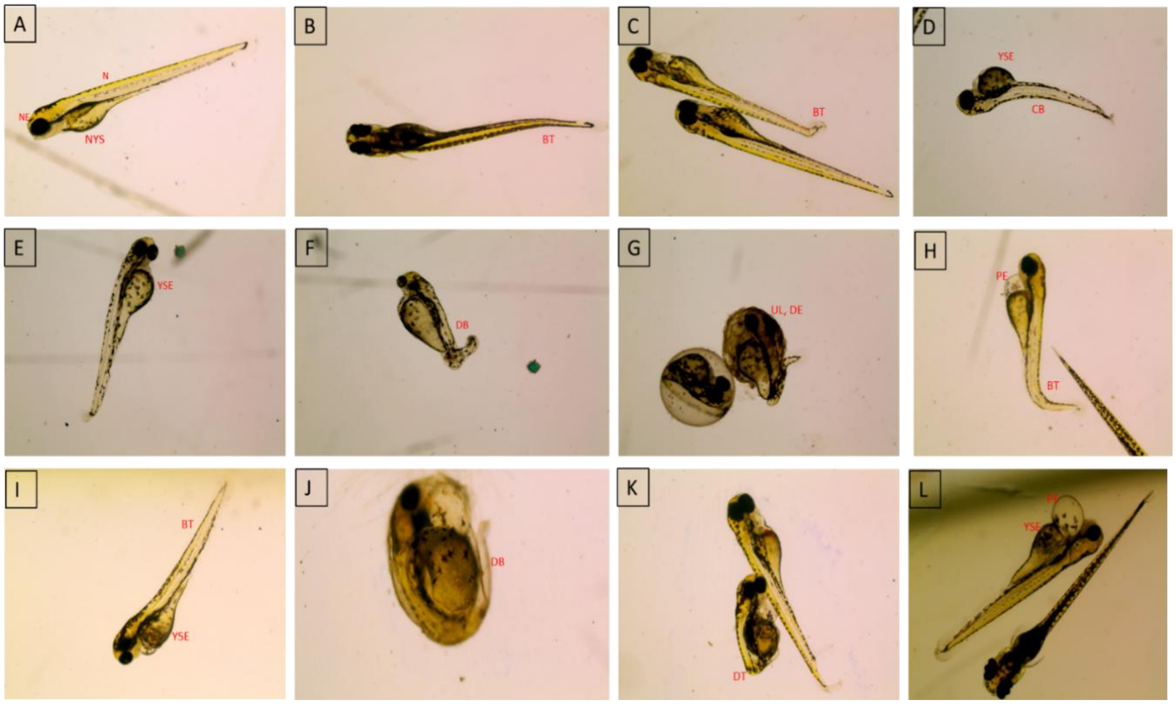

Fig. 2: Photomicrographs showing morphological deformities in zebrafish larvae after 96h of exposure of DHBP (A) Normal larvae; (B-L) DHBP exposed groups. Normal larvae- N, normal eye- NE, normal yolk sac- NYS, bent tail- BT, crooked body- CB, yolk sac edema- YSE, deformed body- DB, unhatched larvae- UL, deformed embryo- DE, pericardial edema- PE, deformed tail- DT. (n=50).

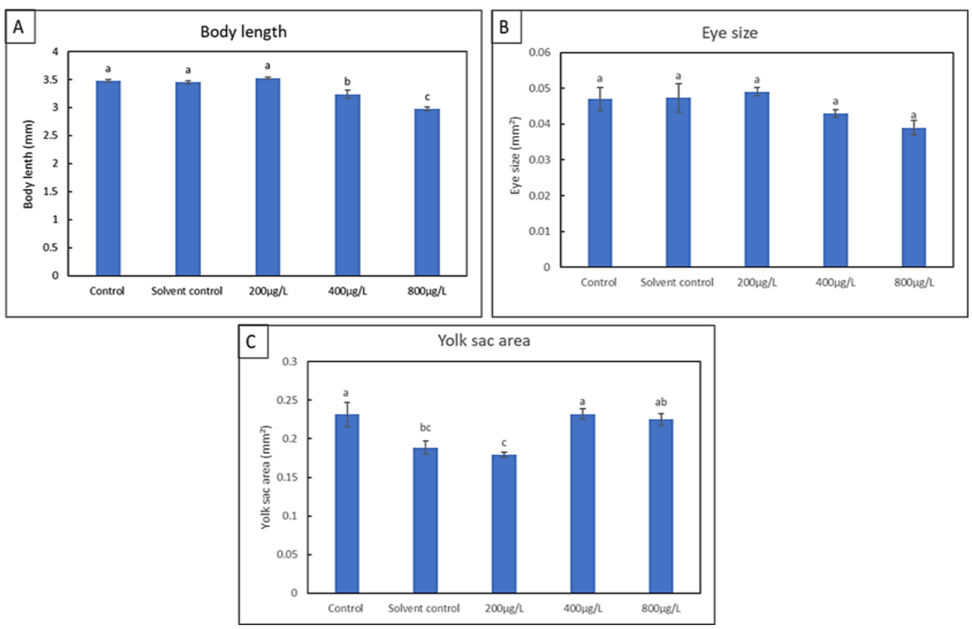

Fig. 3: Effect on body length, eye size and yolk sac area in zebrafish larvae under exposure of DHBP at different concentrations at different same time interval. (A) Body length (B) Eye size (C)Yolk sac area. Different letters a, b, c signifies the effect of different concentrations at 96 hours of exposure. Data represent the mean±SE. (n=50).

4. DISCUSSION

UV-filters (UV-Fs) have emerged as environmental contaminants of great concern in the recent years (Molins-Delgado *et al.*, 2016). UV-Fs constitute a large and heterogenous group of chemicals that are widely used as cosmetic ingredients in several personal care products such as shampoos, soaps, lipsticks, after- shave lotions and sunscreens (body lotions) that offer protection from sunburn. The wide spread application of UV-Fs in personal care products and incomplete removal in waste water treatment plants leads to entry of these compounds into the environment (Balmer *et al.*, 2005). Due to their lipophilic nature, UV-Fs can accumulate in the biota and may cause adverse effects in both aquatic organisms and humans (Quintaneiro *et al.*2019). The toxic effects of organic UV-F Benzophenone have been reported in various aquatic life species in previous years. DHBP (a derivative of benzophenone) is widely used as a UV stabilizer in various consumer products, including plastics and cosmetics, its persistence in the environment and potential for long-term biological effects remain poorly understood. There has been limited prior research focused specifically on 4,4'-Dihydroxybenzophenone, particularly regarding its toxicological impacts on aquatic organisms. So, the present study has been undertaken to assess the toxic effects in zebrafish embryo-larvae after the exposure of DHBP at different concentrations at different time duration.

The present study showed a time and concentration dependent increased mortality rate in the zebrafish embryo-larvae exposed groups of DHBP. A similar increased mortality rate in UV filter BP-3 exposed zebrafish embryos was observed (Bluthgen *et al.*, 2012). Zhang *et al.* (2021) also reported a significantly increased mortality rate in zebrafish embryos when exposed to oxybenzone and benzophenone by UV light. In zebrafish embryogenesis, the hatching of embryos at specific time is considered as an important step. Hatching rate is defined as the ratio of hatching embryos to the total number of embryos exposed at the beginning. In the present study, a significantly decreased hatchability rate was observed in zebrafish embryos exposed to DHBP. Similarly, Bluthgen *et al.* (2014) examine that accumulation of octorylene (OC) in zebrafish embryos caused delayed hatching.

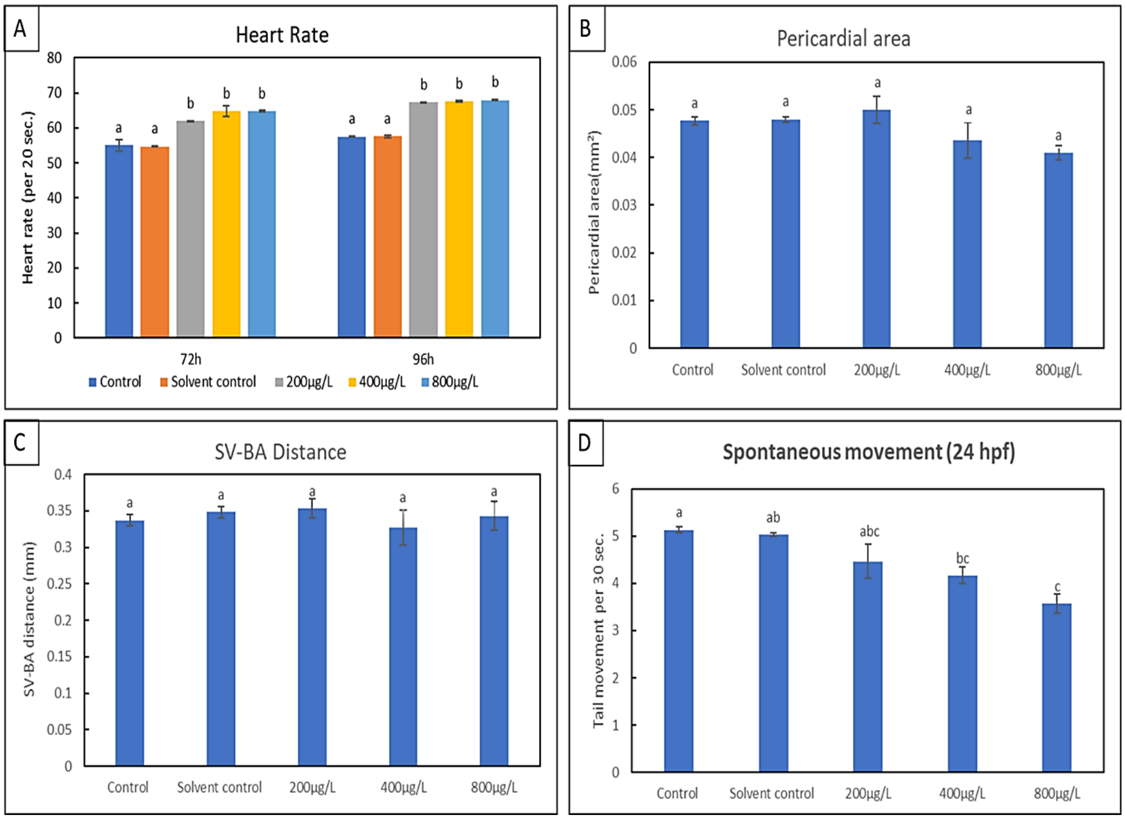

Fig. 4: Effect on heart rate, pericardial area, SV-BA distance and spontaneous movement in zebrafish larvae under exposure of DHBP at different concentrations at different time interval. (A) Heart rate (B) Pericardial area (C) SV-BA distance (D) Spontaneous movement. Different letters a, b, c signifies the effect of different concentrations at different hours of exposure. Data represent the mean±SE. (n=50).

Different types of morphological deformities including bent spine, crooked tail, pericardial edema, yolk sac edema were observed in zebrafish embryo-larvae exposed to different concentrations of DHBP. Similarly, Zhang *et al.* (2021) examined the morphological abnormalities such as pericardial edema, yolk sac edema and bent spine in zebrafish larvae exposed to BP and BP-3. Growth parameters include body length, eye size and yolk sac area, all were examined at the end of experiment. It was observed that body length and yolk sac area was significantly reduced but there was no significant difference observed in eye size of zebrafish larvae exposed to DHBP at different concentrations. Similarly, Zuo *et al.* (2023) reported that larvae exposed to BP showed shortened body length and yolk sac area. Prakash *et al.* (2022) examined that exposure of zebrafish larval stages to 4-methylbenzylidene camphor resulted reduced body length.

In present study, heart rate was observed to be significantly increased but no significant difference was observed in SV-BA distance and pericardial area in zebrafish embryos exposed to DHBP at different concentrations. Similarly, in previous studies Bendrossiantz *et al.* (2023) reported increased heart rate in zebrafish larvae exposed to carbaryl and fenitrothion. Garbinato *et al.* (2020) also examined increased heart rate in zebrafish larvae exposed to ractopamine hydrochloride. The alterations in spontaneous tail movement have been used to evaluate the neurotoxic behavior of zebrafish embryos. Spontaneous tail movement is the first motor activity generated by developing neurons network in zebrafish embryos. In present study, spontaneous tail movement was significantly reduced in zebrafish embryos exposed to DHBP at different concentrations. Similarly, Zindler *et al.* (2019) reported decreased spontaneous tail movement in zebrafish embryos exposed to fluoxetine and citalopram.

5. CONCLUSION

In conclusion, ecotoxicological information on DHBP in aquatic organisms remains limited. The present study demonstrates that DHBP induces developmental toxicity in zebrafish embryo–larvae, evidenced by increased mortality, reduced hatchability, and morphological deformities. Cardiac toxicity was reflected by an elevated heart rate, while SV–BA distance and pericardial area remained unaffected. DHBP also caused neurobehavioral toxicity, indicated by reduced spontaneous tail coiling movements, and affected growth parameters by reducing body length and yolk sac area, with no significant change in eye size. Overall, these findings suggest that DHBP may pose a risk to the normal development of aquatic organisms, highlighting the need for further studies to elucidate its underlying mechanisms and long-term ecological impacts.

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Author Contributions

Pallvi Sharma: Conceptualization, Methodology, Writing – original draft, Data curation. **Shiv Kumar:** Methodology, Writing – review & editing Data curation. **Pooja Chadha:** Supervision, Writing – review & editing.

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Availability of data and materials

Not applicable.

Competing interest

The authors declare no competing interests.

Ethics approval

All the experiments were performed according to the protocol approved by the Institutional Ethical Committee of Guru Nanak Dev University, Amritsar (letter no. 226/CPCSEA/2023/02) for the zebrafish and handled in accordance with animal use protocols (AUPs).

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