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# Toxicity assessment of di(2-ethylhexyl) phthalate using zebrafish embryos: Cardiotoxic potential

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## ABSTRACT

Plasticizers are considered as newly emerged contaminants. They are added to plastics to increase their flexibility and softness. Phthalate plasticizers including the Di-2-ethylhexyl phthalates (DEHP) are toxic and induce adverse effects on the different organization levels of the environment. In the current study, we investigated the potential toxicity of DEHP using Zebrafish as a biological model. Five ascending concentrations of DEHP were tested in embryos throughout 96 hpf: 0.0086, 0.086, 0.86, 8.6, and 86 mg/L. Embryotoxicity assessments revealed limited lethal effects on DEHP-exposed embryos, yet notable anticipation of the hatching process was observed at 48 hpf. Although DEHP showed negligible influence on the length and pericardial area of exposed embryos, it led to multiple bodily deformities. Gene expression analyses of key cardiogenic and inflammatory genes evidenced alterations in *tbx20*, *bcl2*, and *il1b* expression in Zebrafish embryos at 96 h post-fertilization. Results from the cardiac function analysis displayed that DEHP significantly affected the arterial pulse and linear velocity within the Posterior Cardinal Vein (PCV) of exposed fish. These findings strongly advance that even at low concentrations, DEHP can be considered as potential toxic agent, capable of inducing cardiotoxic effects.

## 1. Introduction

Industrial progress is a double-edged sword; it has made our lives easier, whereas this lifestyle that seems to be convenient and practical exposes us to potential health hazards of chemicals that find their way into the environment and present a serious threat to ecological equilibrium as well as public health (Cao et al., 2022). Plastics are at the top of the most common pollutants that require urgent action. Despite many warning signs that have highlighted its impact on the environment, statistical analysis revealed that plastics are now produced at 390.7 million metric tons (Garside, 2024). From the global plastic production, 79 % is discarded in ecological areas (Brown et al., 2019) and due to their durability, they are subject to continuous processes of degradation called “aging processes” covering UV radiation, mechanical abrasion, temperature effects, and biodegradation (Dawson et al., 2018).

Compared to pure plastic polymers, plastic additives including plasticizers are relatively toxic. They are added to plastics during processing to provide softness and increase flexibility of the products (Geyer et al., 2017). Between 80 and 90 % of plasticizers' production is allocated for the manufacturing of plasticized polyvinyl chloride (Jamarani

et al., 2018). Plasticizers are considered newly emerged contaminants because of many reasons. Indeed, plasticizers easily leach from plastics and are difficult to degrade which may explain their abundance in the environment (Lebreton and Andrady, 2019). These additives are mainly composed of phthalate and non-phthalate groups of chemicals. In 2015, phthalates production have been amounted to 8 million tons (Net et al., 2015). Due to their extensive use and tendency to leach from various products, phthalates are globally pervasive, present in both aquatic environments and drinking water (Fromme et al., 2007).

Nearly 50 % of global phthalate consumption is composed of DEHP (Kwan et al., 2021) which is the primary phthalate used in commerce including medical devices (Bourdeaux et al., 2004), plumbing, food products (Wang et al., 2020a), and personal care products (Wassenaar et al., 2017). DEHP is a high molecular weight compound that makes its degradation difficult under natural conditions. In packaging materials, DEHP was found at 319 ng/cm<sup>2</sup> (Sioen et al., 2012). DEHP concentrations in foods were detected at up to 3.41 mg/kg, with the highest mean values of 0.23 mg/kg found in meat and 0.21 mg/kg in vegetable oil (Fierens et al., 2012; Siu, n.d.). In China, DEHP was detected in drinking water and pointed its presence at concentrations reaching 50 mg/L. Due

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to reproductive and developmental toxicity findings across various mammalian species, DEHP has been categorized as a Class 1B substance for reproductive toxicity (Den Braver-Sewradj et al., 2020). Recently, many researchers assessed DEHP impact and predicted that prenatal exposure to DEHP may affect cognition and behavioral development in newborns (Den Braver-Sewradj et al., 2020). In Korea, DEHP was detected in over 80 % of analyzed breast milk samples, showing a median concentration of DEHP metabolites at 2.08 µg/L. This substantial value raises significant health concerns and should be a particular area of focus and concern. In the aquatic environments, DEHP proved its contribution in inducing adverse effects on aquatic organisms of which, reproduction, endocrine-hormone, cardiac, neurological, and neuro-behavior are the main affected systems (Carnevali et al., 2010; Gao et al., 2018; Zhang et al., 2018). Chronic exposures of female Japanese medaka to DEHP displayed significant increased levels of sex hormones (E2 and T), decreased percentages of mature oocytes hence alteration of the fertilization rate (Kim et al., 2001). Very recently, it was shown that DEHP has obesogenic effect. Buerger et al. (2020) overfed Zebrafish with DEHP-contaminated food for 60 days and noted alterations of the carbohydrate metabolism, biosynthesis of fatty acids, and lipid metabolism. It is worth noting that DEHP disrupt also the endocrine mechanism (Jia et al., 2016; Ye et al., 2014) through the thyroid hormone homeostasis (Jia et al., 2016). More deeply, DEHP may act as genotoxic agent (Boran and Terzi, 2019): DNA methylation, DNA strand breaks, and increased frequency of micronucleus are the major parameters providing this potential effect (Boran and Terzi, 2019). Because of large panel of toxicity effects as described above, DEHP is now restricted in all children's toys and child care articles to a mass percentage of 0.1 % by the European Union, Canada, and US (Lucarini et al., 2021). The extensive prevalence of DEHP generated significant attention regarding its impact on cardiovascular diseases (CVDs). Research has unveiled the capacity of DEHP to interfere with cardiac cell signaling, gene expression, and metabolism, intensifying cardiac oxidative stress and provoking an inflammatory reaction (Miller, 2020; Vázquez-Carrera, 2016).

Hence, the objective of this study is to explore the potential cardiotoxic impact of DEHP at different concentrations using Zebrafish embryos as a biological model. Our approach involved: 1) the evaluation of the embryotoxic effects of DEHP by the determination of LC<sub>50</sub>, hatching rates (%), body deformities, body length, and pericardial area, 2) the examination of the expression of genes related to both cardiogenesis and inflammatory response, 3) the assessment of the cardiac function of DEHP-exposed fish by measuring parameters such as blood flow, arterial pulse, and linear velocity. This multi-tiered approach aimed to comprehensively understand the potential adverse effects of DEHP on cardiac development and function in the Zebrafish embryo models.

## 2. Materials and methods

### 2.1. Chemicals and reagents

In the present study, Di-2-ethylhexyl phthalate (DEHP; cat#36735) was purchased from Sigma Aldrich (USA). The medium “egg water” used for the growth of Zebrafish embryos was prepared by mixing the following components: 17.53 g of 5.0 mM Sodium Chloride (NaCl), 0.76 g of 0.17 mM Potassium Chloride (KCL), 2.37 g of 0.6 mM Magnesium Sulfate (MgSO<sub>4</sub>)·7H<sub>2</sub>O, 3.53 g of 0.4 mM Calcium Chloride-H<sub>2</sub>O (CaCl<sub>2</sub>·H<sub>2</sub>O), and 1 L of deionized distilled MilliQ water (ddH<sub>2</sub>O). PureLink™ RNA Mini Kit (cat#12183025), SuperScript™ IV VILOTM Master Mix (cat#11756050), and SYBR Green Supermix (cat#1725271) were purchased from ThermoFisher Scientific, Invitrogen, and BioRad, respectively.

### 2.2. Zebrafish maintenance and exposure

Wild-type adult Zebrafish are maintained in the Zebrafish facility

within Qatar University's Biomedical Research Center, under strictly controlled laboratory conditions. These conditions encompass a consistent photoperiod of 14 h of light and 10 h of darkness, along with a constant water temperature set at 28 °C. All husbandry and spawning procedures followed the protocols approved by Qatar University's Animal Care and Use Committee (QU-IACUC). As Zebrafish embryos used in the present work were no >96 hpf, approval from the Institutional Biosafety Committee (IBC) for the use of Zebrafish embryos was deemed sufficient (Approval ID: QU-IBC-2023/041). Following natural spawning, high quality fertilized embryos (SR > 95 %) were selected and exposed to DEHP starting at 2 hpf. Concentrations of acute exposure solution were set at six gradients: 0.008; 0.086; 0.86; 8.6; and 86 mg/L, with a negative control (0 mg/L) exposed to DEHP-free egg water. The highest concentration chosen in the present study corresponds to DEHP water solubility concentration (86 mg/L) as specified in the manufacturer's sheet. In six-well plates, 180 Zebrafish embryos were divided into 6 groups, with each group containing 30 embryos. Within each group, triplicate experiments were conducted, resulting in three batches for each selected concentration. In each well plate, 3 mL of media containing plasticizers at varying concentrations was used to house 10 Zebrafish embryos per well. The experiment was conducted over a total period of 96 h post-fertilization (hpf) during which, the half-exposure solutions were replaced daily to maintain the freshness of the contaminant in the exposure medium. Every 24 h, measurements of the mortality rate (%) and hatching rate were taken, and any deceased embryos were automatically removed from the wells to prevent interference.

### 2.3. Embryotoxicity assessment

The embryotoxicity test was assessed every 24 h under a ZEISS stereomicroscope by calculating the percentages of mortality rate (%), hatching rate (%), and deformity rate, and imaging the body deformities in DEHP-exposed fish. In addition, measurements of the body length and pericardial area were determined as the distance from the foremost point of the head to the farthest end of the tail.

### 2.4. RNA isolation and qPCR analysis

After treatment with DEHP at different exposure concentrations, 96 hpf embryos were sacrificed by an overdose of MS-222 (0.3 %). Samples were preserved in RNAlater and stored at 4 °C until RNA extraction. For each exposure concentration, 20 tested embryos were pooled due to the minuscule size of Zebrafish embryos. This precaution was taken to mitigate the risk of obtaining minimal RNA quantities during experimentation. Total RNA was extracted using a PureLink™ RNA Mini Kit (cat#12183025) following the manufacturer's instructions. RNA concentration was determined at 260 nm using a Nanodrop (Thermo Scientific) and RNA samples with a ratio of absorbance (260/280 nm) ranging from 1.8 to 2.0 were considered suitable for cDNA strand synthesis. 350 ng of RNA was reverse-transcribed into cDNA using SuperScript™ IV VILOTM Master Mix. In QuantStudio 6 Flex system (Applied Biosystems, USA), RT-qPCR was carried out using SYBR Green Super-Mix. The standard cycling conditions considered for all the target genes were set at 50 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The melt curve stage conditions were 95 °C for 15 s and 60 °C for 15 s. Relative expression of target gene mRNA was determined by the ΔΔCt method, normalized to *elf1a* levels. The primers of genes sequences are listed in Table 1.

### 2.5. Heart function analysis

At 96-hpf, heart function analysis was conducted on nine embryos per exposed group, and the results were compared to controls. The technique employed MicroZebraLab Blood Flow from Viewpoint (version 3.4.4, Lyon, France), following the protocol optimized by Benslimane et al. (2019). High-speed time-lapse movies of the tail in

**Table 1**  
Primer pairs of target genes in RT-qPCR analysis.

Genes	Primers	Sequence
<i>tbx20</i>	Forward	5'-GCACTCATGTCAAGTGGGA -3'
	Reverse	5'-CGAGTTTGGATGGCATGA-3'
<i>gata4</i>	Forward	5'-CCAGTCTGCAACGCATGTG-3'
	Reverse	5'-GATCGCCGACTGACCTTCAG-3'
<i>gata5</i>	Forward	5'-GGGACGCCAGGGAACCTA-3'
	Reverse	5'-CACGCGTTGCACAGGTAGTG-3'
<i>gata6</i>	Forward	5'-AGTCGCGACCACTACCTTTCAA-3'
	Reverse	5'-CCTTCGGGATTGCAGTGAGT-3'
<i>tbx5</i>	Forward	5'-CGGATGTTTCCGAGCTTCAA-3'
	Reverse	5'-CATCGCAGGCTCAGCTTTC-3'
<i>bcl2</i>	Forward	5'-TCACTCGTTCAGACCCCTCAT-3'
	Reverse	5'-ACGCTTTCACGCACAT-3'
<i>il1b</i>	Forward	5'-CATGCGGGCAATATGAAGTC-3'
	Reverse	5'-AAACGAGCCTGGCTGTAAGG-3'
<i>ef1a</i>	Forward	5'-TGGTGGTGTGCGTGAGTTTG-3'
	Reverse	5'-AAACGAGCCTGGCTGTAAGG-3'

1000 frames per 10 s at 100× magnification were recorded to measure the cardiac parameters in PCV including blood flow, arterial pulse, and linear velocity.

## 2.6. Statistical analysis

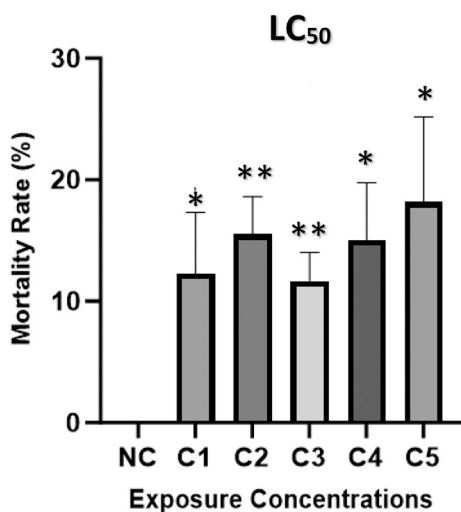
For all the experiments conducted in the present study, the statistical analysis was determined using GraphPad Prism 10 Statistical program using Dunnett's test after One Way Anova. The Shapiro–Wilk test was performed to test the normality of the data. Results are given as Mean ± Standard Deviation;  $p < 0.05$  was considered significant (\*),  $p < 0.01$  was considered highly significant (\*\*), and  $p < 0.001$  was considered very highly significant (\*\*\*).

## 3. Results

### 3.1. Embryotoxicity assessment

#### 3.1.1. LC<sub>50</sub> determination

The LC<sub>50</sub> in DEHP-exposed embryos was determined at 96 hpf through the calculation of the mortality rate (%) as represented in Fig. 1. For each exposure concentration, mortality rates (%) in DEHP-exposed groups were compared to controls that did not display any dead embryo throughout the experiment. Our findings revealed that the highest



**Fig. 1.** LC<sub>50</sub> determination in DEHP-exposed embryos at different exposure concentrations. Results are represented as mean ± SD (Dunnett's test: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

mortality rate among fish occurred at the highest concentration (C5) of DEHP, leading to the mortality of 18.8 % of the tested fish. In terms of lethal concentration, while LC<sub>50</sub> was not specifically determined, we concluded LC<sub>18</sub> from the exposure of Zebrafish embryos to the highest concentration of DEHP (C5).

#### 3.1.2. Hatching rate (%)

Despite the fact that all exposed embryos were hatched by the end of the experiment, the hatching rate (%) was hasted in all DEHP-exposed groups at 48 hpf when compared to controls (Table 2). This acceleration in the hatching process was detected at 13.37 %, 20.63 %, 22.19 %, 20.4 %, and 10.7 % in C1, C2, C3, C4, and C5 treated groups, respectively.

#### 3.1.3. Body deformities

As displayed in Fig. 2I, the entire body, heart, yolk sac, and tail were all examined for defects. In NC groups, the whole body appeared intact without any observed deformities (Fig. 2 I.A and II). At the lowest concentration (0.0086 mg/L), yolk sac edema and absence of tail were observed with a rate of 2.5 % (Fig. 2 I-B and II). At 0.086 mg DEHP/L, 3.12 % of DEHP-exposed fish displayed pronounced pericardial edema (Fig. 2 I-C and II). Indeed, tail flexure, yolk sac edema, and necrosis were the main observed changes in 2.77 % of Zebrafish embryos exposed to 0.86 mg DEHP/L (C3) (Fig. 2 I-D and II). At 8.6 mg/L, lordosis and yolk edema were observed with a DR of 2.5 % in DEHP-exposed embryos (Fig. 2 I-E and II) however, at the highest concentration (86 mg/L), lordosis was the unique change observed in 3.12 % of DEHP-exposed fish (Fig. 2 I-F).

#### 3.1.4. Body length

In DEHP-exposed groups, the body length was calculated and compared to controls at 96 hpf (Fig. 3). The average of body length in control groups was 3.47 mm. In DEHP-exposed groups, the body length of all embryo samples did not display significant changes ( $p > 0.05$ ) indicating that exposure to DEHP does not significantly influence the body length of embryos at 96 hpf.

#### 3.1.5. Pericardial area

The pericardial area in DEHP-exposed fish was measured and compared to controls at 96 hpf. As displayed in the body length results, data from the pericardial area measurements demonstrated that DEHP exposure did not significantly affect the size of the pericardial area in exposed fish compared to controls ( $p > 0.05$ ).

Overall, the different exposure concentrations of DEHP had no significant effects on the general morphology of Zebrafish embryos after 96 h of exposure to the chemical.

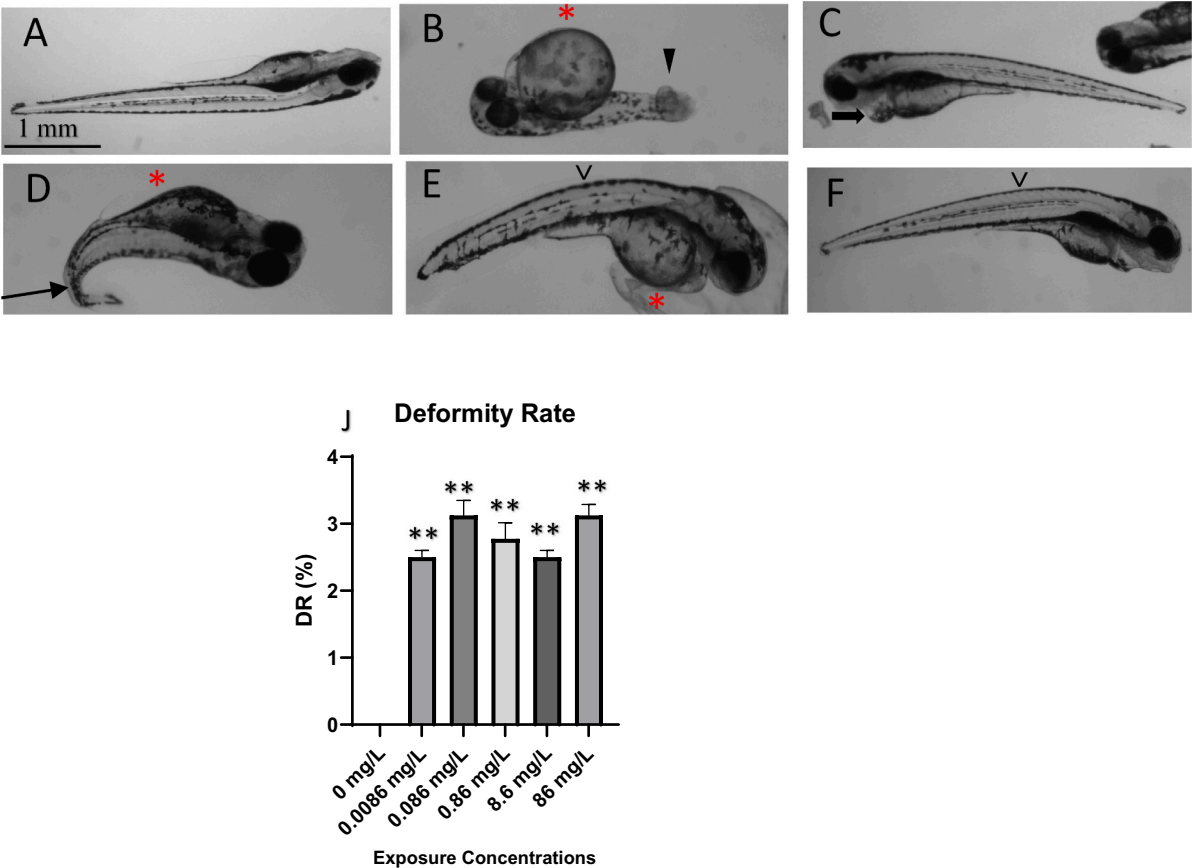
### 3.2. Gene expression

Fig. 4 illustrates the relative expression profiles of key cardiac gene biomarkers (*gata4*, *gata5*, *gata6*, *tbx5*, *bcl2*, *tbx20*, and *il1b*) in Zebrafish embryos exposed to DEHP at different concentrations. Throughout the entire control group experiment, the selected gene expressions remained stable and did not exhibit significant changes. In DEHP-exposed

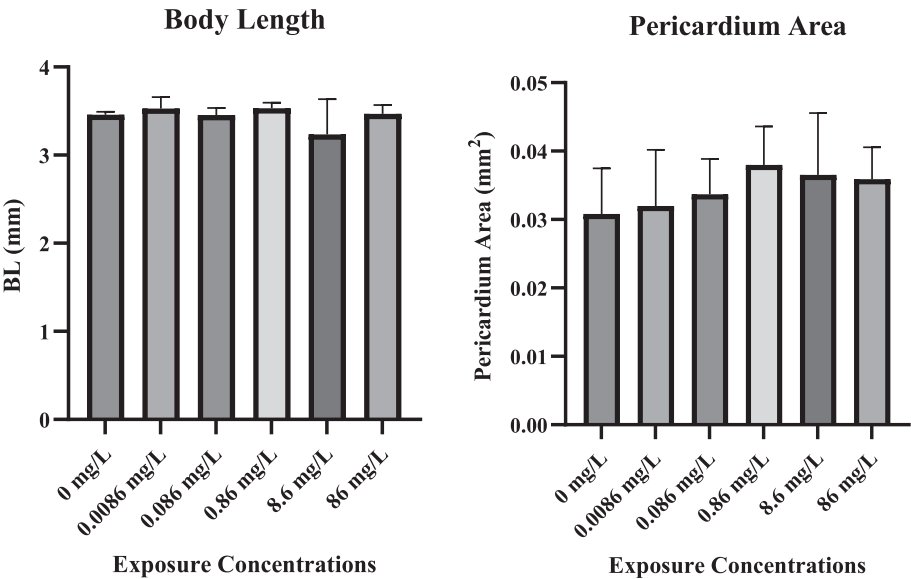
**Table 2**

Hatching rate (%) of Zebrafish embryos at different time points of the experiment. Results are represented as mean ± SD (Dunnett's test: <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ , <sup>c</sup> $p < 0.001$ ).

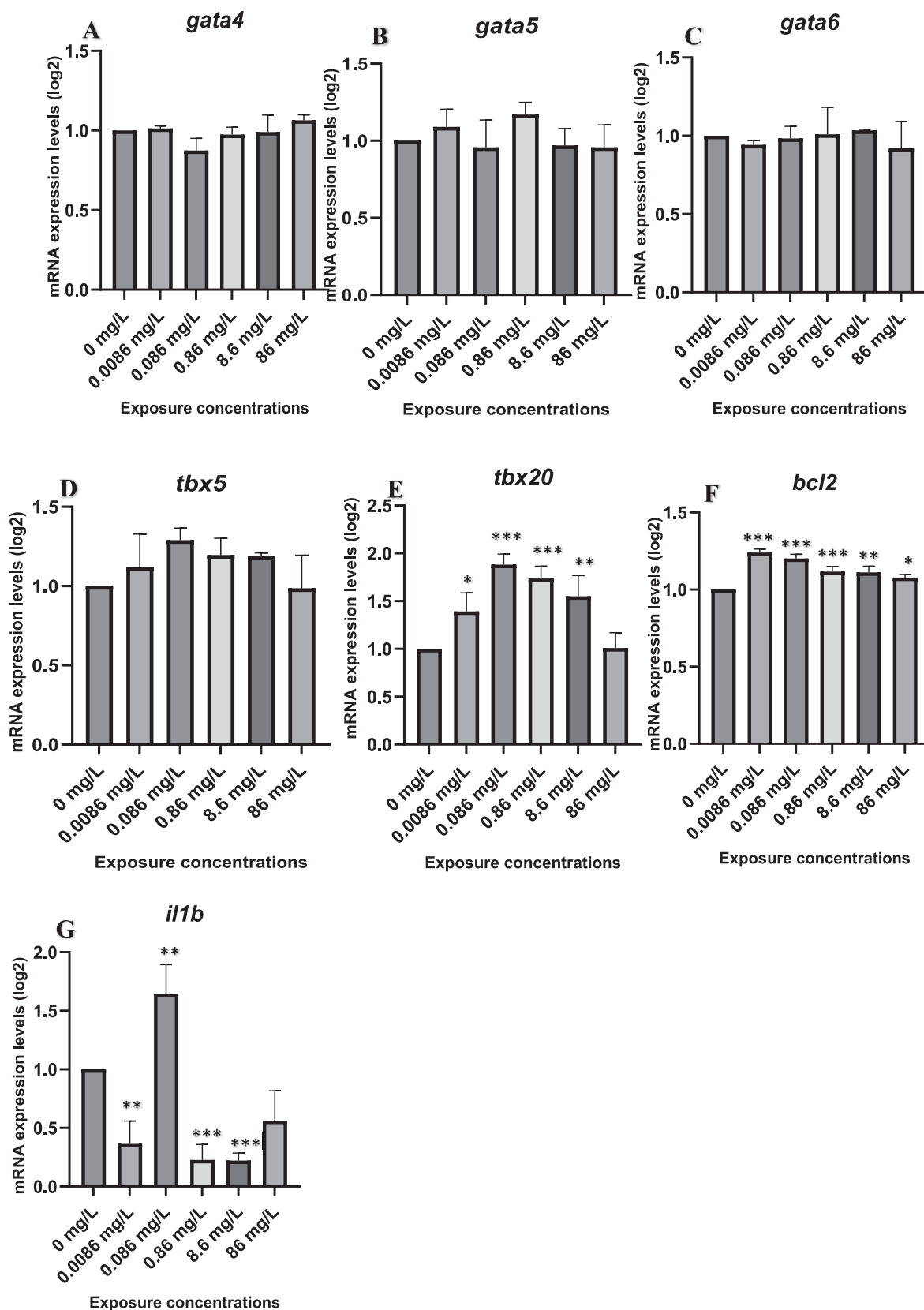
	24 h	48 h	72 h	96 h
C0	0	51.66 ± 26.87 <sup>b</sup>	100	100
C1	0	65.03 ± 15.09 <sup>c</sup>	100	100
C2	0	62.32 ± 32.91 <sup>b</sup>	100	100
C3	0	73.85 ± 15.42 <sup>b</sup>	100	100
C4	0	62.2 ± 2.69 <sup>c</sup>	97.5 ± 5 <sup>c</sup>	100
C5	0	62.34 ± 21.7 <sup>b</sup>	100	100



**Fig. 2.** Whole body images and DR (%) of control and DEHP-exposed embryos ( $N = 30$ ) at 96 hpf (I and II). I: The whole body of control fish displayed a normal morphology (A). All treatments (B to F) displayed injuries including pericardial edema ( $\Delta$ ), yolk sac edema (\*), lordosis (v), tail flexure ( $\rightarrow$ ), absence of tail ( $\emptyset$ ), and necrosis ( $\square$ ) (see text for details). All images were observed under stereomicroscope at  $2.0\times$ . II: DR (%) of Zebrafish embryos calculated at 96 hpf. Results are represented as mean  $\pm$  SD (Dunnett's test: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).



**Fig. 3.** Body Length and Pericardium Area measurements in Zebrafish embryos (10 embryos/concentration) in triplicates at 96 hpf upon exposure to DEHP at different concentrations (0; 0.0086; 0.086; 0.86; 8.6; and 86 mg DEHP/L). Values that are significantly different from controls are indicated by asterisks (one-way ANOVA, followed by Dunnett's test: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). Values are presented as mean  $\pm$  SD.



**Fig. 4.** Relative mRNA expression in Zebrafish embryo controls and DEHP-exposed groups at different exposure concentrations for *gata4* (A), *gata5* (B), *gata6* (C), *tbx5* (D), *tbx20* (E), *bcl2* (F), and *il1b* (g). The ordinate represents the Log2 relative expression determined by the comparative Ct method. Each bar represents mean  $\pm$  SD. \* denotes significant differences to controls for a given exposure concentration (Dunnett's test, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).



embryos, expressions of the selected genes were variable. Exposure to DEHP did not induce significant changes in the expression of *gata4*, *gata5*, *gata6*, and *tbx5* across the exposure period (Fig. 4 A, B, and C). However, a significant effect of DEHP on *tbx20* expression was observed at 0.086, 0.86, and 8.6 mg DEHP/L (Fig. 4D). Indeed, the highest upregulation of *tbx20* mRNA occurred at 0.086 mg DEHP/L, reaching 1.82-fold the expression of controls ( $p < 0.001$ ). Regarding *bcl2*, the expression was highly affected by the exposure to different DEHP concentrations. *bcl2* was up regulated in all DEHP-tested groups. On the other hand, mRNA expressions of *il1b* varied across the exposure concentrations. Significant upregulation of the gene expression was observed at 0.086 mg DEHP/L ( $p < 0.05$ ), while downregulation was evident at 0.0086, 0.86, 8.6, and 86 mg DEHP/L ( $p < 0.05$ ).

### 3.3. Heart function analysis

The results pertaining to Zebrafish embryo heart function measured in PCV are presented in Fig. 5. Throughout the entire exposure duration to DEHP, blood flow, arterial pulse, and linear velocity of control fish remained consistent in PCV, exhibiting no observable changes ( $p > 0.05$ ). Analyzing the blood flow in DEHP-exposed group hearts (Fig. 5A) revealed that DEHP has no effects on blood flow in PCV. However, DEHP significantly increased the arterial pulse in PCV of DEHP-treated embryos, reaching its highest rate at the highest exposure concentration (86 mg DEHP/L) (Fig. 5B). In Fig. 5C, a remarkably significant reduction in the linear velocity within the PCV was evident upon exposure of Zebrafish embryos to 0.086 mg DEHP/L. This reduction was measured at a substantial 4.03-fold decrease compared to controls ( $p < 0.001$ ).

## 4. Discussion

DEHP is regulated by the Environmental Quality Standards Directive of the EU Water Framework Directive as a priority substance because of its toxic potential (EC (European Commission), 2008). This plasticizer is the most used plasticizer in industries including medical devices (Bourdeaux et al., 2004), plumbing, food products (Wang et al., 2020b), and personal care products (Wassenaar et al., 2017). In the present study, we investigated the potential cardiotoxic effect of DEHP using Zebrafish embryos. A series of six ascending concentrations of DEHP were tested as follows: 0, 0.0086, 0.086, 0.86, 8.6, and 86 mg/L.

### 4.1. DEHP-exposure concentration did not reach $LC_{50}$ at 96 hpf

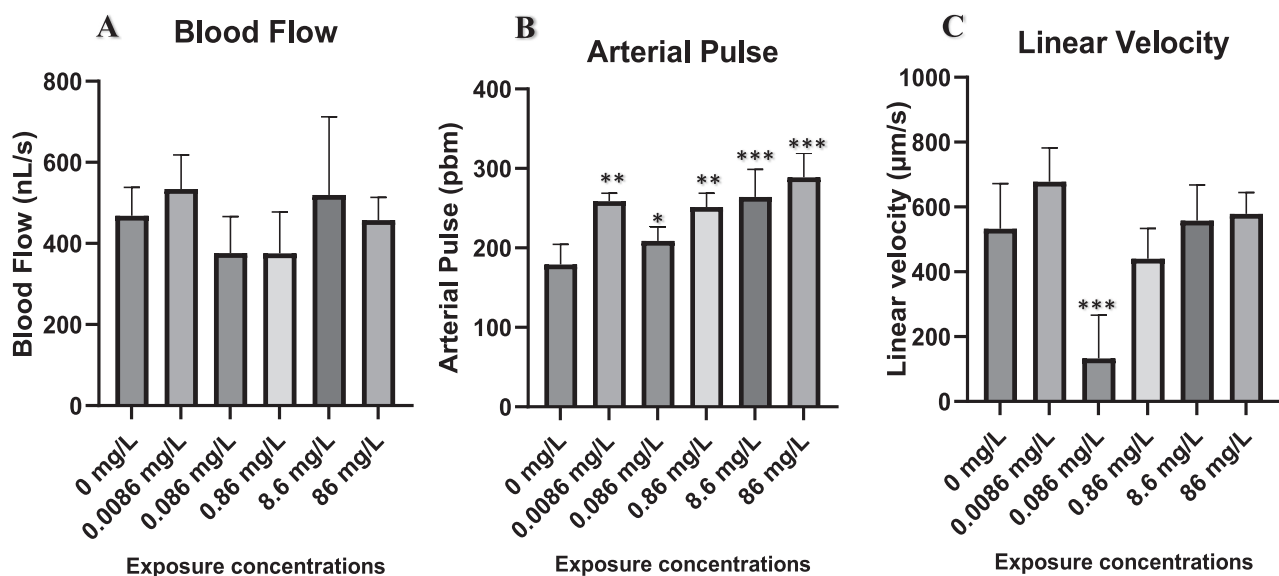
Our findings from the toxicity tests showed that DEHP has a low lethal effect on Zebrafish where the mortality rate did not exceed 18.8 % in embryos at 96 hpf. Similar studies also reported that DEHP has minimal lethal effects on exposed fish. At 50  $\mu$ g/L of DEHP, the mortality rate of Zebrafish embryos was detected at 19.5 % (Pu et al., 2020). When Zebrafish embryos were injected with 0.02 g DEHP, no significant difference was found elsewhere in embryonic lethality between control and DEHP groups at 24 hpf (Sun et al., 2021a). Additionally, Mansuri et al. (2023) reported that DEHP did not significantly affect the survival of Zebrafish embryos.

### 4.2. DEHP accelerated the hatching process

In the present work, analysis of the hatching rate performed in all tested groups displayed that DEHP affected the hatching process of Zebrafish. Compared to controls, the hatching rate (%) was significantly accelerated at 48 hpf in DEHP-exposed fish to all exposure concentrations. It is well known that chorions protect the embryo from exposure to external agents. Accelerated hatching rate (%) in DEHP-exposed groups may increase the potential toxic effects of DEHP even at the lowest concentration (0.0086 mg DEHP/L). In our knowledge, this is the first study conducted on DEHP showing that the chemical accelerated the hatching process in exposed fish. Üstündağ et al. (2017) revealed that exposure of Zebrafish embryos to 2.5  $\mu$ g DEHP/L inhibited the hatching after 48 and 72 hpf. Likewise, Wang et al. (2022) showed that DEHP decelerated the process of hatching in Zebrafish exposed to 0.071 mg/L over 9 days of exposure.

### 4.3. DEHP caused morphological defects

Despite DEHP not swaying the body length and the pericardial area of treated fish, each concentration of DEHP induced body deformities, albeit with a low occurrence rate. Absence of tail, pericardial and yolk sac edema, lordosis, and tail flexure were the most detected changes in DEHP-exposed embryos. These changes can be attributed to the anticipated hatching event observed in all DEHP-treated embryos at 48 hpf. Similar results were reported by McCollum et al. (2011) and Tao (2023) who demonstrated that embryonic exposure to DEHP induced yolk sac



**Fig. 5.** Heart function analysis of Zebrafish control and DEHP-exposed at 96 hpf. Blood flow (A), arterial pulse (B), and linear velocity (C) are the heart function parameters analyzed in all tested embryos. Each bar represents the mean  $\pm$  SD of 9 individual fish. \* denotes significant differences to controls for a given time exposure (Dunnett's test, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

abnormalities. In addition, Mu et al. (2018) found that 250 µg/L of DEHP significantly induced yolk sac edema and increased ratios of yolk sac area to fish body area in DEHP-exposed embryos at 48 hpf. In many other studies, pericardial edema was the most prevalent effect of DEHP observed in Zebrafish treated-embryos. Boran and Terzi (2019) detected pericardial edema in Zebrafish from 72 to 168 hpf after treatment with 10 mg DEHP/L. Furthermore, serious heart looping disorder were observed in DEHP-treated Zebrafish, mainly manifested with an elongated atrialventricular distance (Sun et al., 2021b). According to Mu et al. (2020), the phenotypic changes observed in Zebrafish embryos upon exposure to DEHP may be due to the transcriptional alteration of genes involved in developmental pathways, which might be associated with modified DNA methylation.

#### 4.4. DEHP affected the cardiogenesis and heart function

It is widely believed that environmental pollution leads to the progressive deterioration of cardiac structure and function (Pu et al., 2020). In the present study, we examined the expression of genes involved in the gastrulation completion (*gata4*, *gata5*, *gata6*, and *tbx5*), cardiomyocytes maturation and heart regeneration (*tbx20*), apoptosis (*bcl2*), and inflammation response (*il1b*). To our knowledge, no prior studies have delved into these specific genes or parameters pertaining to heart formation and function, as utilized in our research.

The formation of the cardiogenic region occurs during the early embryonic stages called “gastrulation” mainly controlled by *gata4*, *gata5*, *gata6*, and *tbx5* as indicated by Song et al. (2022). Our findings indicated that exposure to DEHP did not alter the expression of these genes at any tested concentration. Initially, this suggests that DEHP may not have an impact on the completion of gastrulation in Zebrafish hearts. Analyzing both the transcriptional and translational levels may provide further insight into confirming this hypothesis. Therefore, studying the corresponding genes at the protein level is necessary. Our results are not consistent with those of many other studies that revealed an increased susceptibility of DEHP to induced fetal cardiac malformation mainly by inhibiting Gata4/Mef2c/Chf1 during embryogenesis (Song et al., 2022). In the other hand, Mu et al. (2020) analyzed differential gene transcriptions in Zebrafish embryos upon exposure to DEHP. The authors displayed that *nppa*, *my17*, *tbx5b*, *smyd2b*, *klhl41a*, *cntt* and *cmlc1* were altered. However, this subject is open to research, as it is possible that different doses and different exposure durations may cause different results.

Apoptosis plays a critical role in regulating homeostasis and tissue remodeling during the developmental stages (Trede et al., 2004). To prevent apoptosis, anti-apoptotic proteins such as *bcl2* are synthesized in order to regulate and mediate the balance between survival and apoptosis in cells (Giral et al., 2016). In the present work, exposure to DEHP significantly affected the expression of *bcl2* at all the exposure concentrations (ranging from 0.0086 to 86 mg DEHP/L), suggesting that DEHP could be considered an apoptosis-inducer in Zebrafish embryos. In other studies, DEHP was confirmed to induce apoptosis and alter apoptosis-related genes in Zebrafish embryos. For example, Fang et al. (2018) and Parrotta et al. (2020) demonstrated that DEHP induced apoptosis and cell cycle arrest in a model of cardiovascular disease mechanisms mainly by increasing expressions of peroxisome proliferator-activated receptor (PPAR) $\gamma$ . Similarly, Mu et al. (2020) treated 72 h-Zebrafish embryos with 250 µg/L DEHP and observed that the plasticizer induced intense apoptotic signals in the heart region.

*tbx20* expression is a critical determinant in cardiomyocyte cell proliferation and lineage maturation in embryonic cardiomyocytes (Chakraborty and Yutzey, 2012). The gene also promotes heart regeneration in response to various sources of cardiac damage (Fang et al., 2018). Our results displayed that DEHP exposure significantly affected the expression of *tbx20* at all the exposure concentrations except at 86 mg DEHP/L. These findings suggest the possibility of cardiac damage occurring in Zebrafish embryos exposed to DEHP that may be

regenerated by the over-expression of *tbx20*. According to Chakraborty and Yutzey (2012), increased or decreased *tbx20* function in cardiomyocytes can lead to pleiotropic downstream effects on cell proliferation, lineage maturation, or conduction mediated by Tbx20 and its complex interacting regulatory factors.

The inflammatory response serves as a significant mechanistic pathway mediating the adverse CV effects of DEHP. *il-1 $\beta$*  plays a pivotal role in preserving cutaneous homeostasis. Changes in the transcriptional levels of these molecules represent a characteristic aspect of the immune response. Upon investigating the potential effects of DEHP in inducing inflammatory reactions, our results revealed an enhancement in the expression of *il1b* that was over-expressed in all the exposure concentrations. Our finding revealed that DEHP might possess inflammatory properties leading to the induction of *il1b* in treated groups. It was largely demonstrated that DEHP induces inflammatory responses. For example, DEHP treatment induced *icam-1*, *il-6*, and *il-8* overexpression (Wang et al., 2020b). Mansuri et al. (2023) reported that suppression of *il6* gene expression leads to myocardial hypertrophy. Based on these findings, Mu et al. (2018) inferred that DEHP-induced immune response might be a result of disruption of lipid homeostasis.

#### 4.5. DEHP affected the heart function

Cardinal veins are among the first formed vessels during embryogenesis. In the present study, the heart function parameters are the first parameters to be used to assess the cardiotoxic effects of DEHP. Our results displayed that exposure of Zebrafish embryos to all exposure concentrations of DEHP significantly increased the Arterial Pulse in PCV of exposed fish suggesting that DEHP may induce cardiovascular injuries later in the life of Zebrafish. The alteration in heart function in PCV could be linked to the up-regulation of *tbx20*. This up-regulation might prompt increased cell proliferation in the dorsal aorta, thereby accelerating the Arterial Pulse in PCV. However, exposed embryos to 0.086 mg/L of DEHP exhibited a very high significant inhibition of the Linear Velocity in PCV that may generate multiple issues in the passage of Blood Flow from PCV to the heart. Our findings suggest that disturbances that occurred in PCV could be related to pericardial edema observed in DEHP-exposed groups at 0.086 mg DEHP/L. Substantiating this hypothesis necessitates an in-depth analysis. In many other studies, the most common cardiac parameter tested is the heartbeat, which has been widely used as an endpoint for assessing heart toxicity. Moreover, DEHP-induced fetal cardiac malformations in Zebrafish larvae have also been observed in recent studies. DEHP exposure at high concentrations of 120–200 µg/mL decreased the heart rate of embryonic Zebrafish, indicating the developmental risks of DEHP exposure on the CV system (Mu et al., 2020). Posnack et al. (2015) reported that DEHP doses in the µg/L range could markedly decrease the spontaneous beating rate in Zebrafish embryos (Posnack et al., 2015).

### 5. Conclusion

Our study is the first to demonstrate that exposure to DEHP accelerates the hatching process in Zebrafish embryos, potentially increasing the toxic effects of DEHP even at the lowest tested concentration. This novel finding emphasizes the crucial importance of considering impact of DEHP on developmental processes. Our research is also among the first to explore the effects of DEHP on specific cardiac function parameters, such as blood flow, arterial pulse, and linear velocity in PCV of Zebrafish embryos. These parameters, along with the examination of key genes involved in various cardiac functions and inflammatory responses, provide new insights into the potential cardiotoxic effects of DEHP. Notably, our findings reveal that even at low concentrations commonly found in aquatic environments, DEHP can induce cardiotoxic effects. This underscores the urgent need for biomonitoring and assessment of the impact of plasticizers, even at environmentally relevant concentrations, to ensure the protection of aquatic life and the overall health of



our ecosystems.

## CRediT authorship contribution statement

**Azza Naija:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Conceptualization. **Yoshifumi Horie:** Writing – review & editing. **Sonia Boughattas:** Writing – review & editing. **Sara Ismail:** Methodology. **Nafja Al-Mansouri:** Methodology.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Azza Naija reports financial support was provided by Qatar National Library. Azza Naija reports a relationship with Qatar National Library that includes: If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

the authors are allowed to share the publication data if the paper is accepted for publication

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