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Original Article

Combined Effects of Temperature Changes and Bisphenol A on the Embryonic Development of Zebrafish (*Danio rerio*)

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Abstract: Water pollution by heat and organic matter is a global concern. Bisphenol A (BPA), an organic synthetic compound which is used as a raw material in the manufacture of many plastic products. It has been found in water and has the potential to bioaccumulate and disrupt the endocrine system. In this study, the simultaneous influence of BPA and temperature changes on zebrafish embryos (*Danio rerio*) was evaluated. Embryos (0 day) were exposed to BPA (2.5 - 25 mg/L) at three temperature regimes: 23 °C, 28 °C (control) and 33 °C. The amount of embryonic deaths, malformations (hematoma, pericardial edema) were recorded at 24, 48, 72, and 96 hours post-fertilization (hpf). The results showed that embryo mortality and malformation rate increased depending on BPA concentration and temperature. At 96 hpf endpoint, lethal concentration (LC_{50}) and morphological effective concentration (mEC_{50}) of 23 °C, 28 °C, 33 °C were obtained as follows: LC_{50} = 14.886 mg/L and EC_{50} = 10.421 mg/L; LC_{50} = 16.732 mg/L and EC_{50} = 9.336 mg/L; LC_{50} = 7.627 mg/L and EC_{50} = 7.731 mg/L, respectively. The toxicity of BPA at 33 °C was higher than that of the control condition indicating the synergistic effect of BPA and temperature changes on the embryonic development of zebrafish. This suggests that the climate change might also effect on the toxicity of BPA.

Keywords: Bisphenol A, Danio rerio, Water pollution, Organic compound, Climate change.

1. Introduction

Bisphenol A (BPA) is a widely used organic compound in the manufacturing of polycarbonate plastics and epoxy resin, commonly found in products such as baby bottles, medical devices, thermal paper, and food can linings [1-3]. With annual production exceeding 3.8 million tons, BPA is among the most produced chemicals globally, leading to significant environmental contamination

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through wastewater, plastic degradation, and landfill leaching [4]. The U.S. Environmental Protection Agency reports over one million pounds of BPA released annually [5]. It has been detected in various environments, including water [6-8], soil [9, 10], air [4, 11, 12], and even human bodies, with a study showing its presence in 93% of Americans' urine, sweat, and serum of individuals exposed to BPA [13]. A publication in 2015 reported that the average BPA concentration in aquatic ecosystems in Hanoi ranged from14-22 µg/L [14]. Moreover, Guo et al., [15] reported evidence of biomagnification of BPA-related residues in the food chain between algae and rotifers. BPA is also recognized as an endocrine-disrupting chemical (EDC) due to its ability to bind to estrogen receptors [16] and then disrupt normal gene regulation related to estrogen expression, as evidenced in the studies on rats [17] and zebrafish [18].

Temperature, a critical abiotic factor, significantly influences organisms' metabolism, growth, and reproduction, especially poikilotherms [19]. Studies have shown that low and high temperatures can negatively affect physiological responses in marine medaka larvae, increasing mortality and altering enzyme activities [20].

Although some research has examined the individual effects of temperature or BPA, the combined impact of these factors is less understood [20]. Elevated temperatures can exacerbate the release of BPA from products like polycarbonate bottles into the environment [21]. A study in 2022 reported the BPA migration level of 0.089 mg/kg from canned shrimp, exceeding the specific migration limit (SML) of BPA (0.05 mg/kg) specified by the European Union (EU) [22]. Little et al., [23] explored BPA's effects under different thermal conditions but focused on adult zebrafish [23]. Animals often respond to heat stress or toxin exposure by relocating to more suitable habitats [24]. However, early-life stages, such as zebrafish embryos, are particularly vulnerable to environmental changes due to their limited mobility. Therefore, our research investigates the acute effects of temperature and BPA on zebrafish embryos, a model organism commonly used in toxicity studies. This study aims to provide insights into the synergistic effects of temperature and BPA, contributing to a better understanding of their combined impact on aquatic life.

2. Experimental

2.1. Chemicals

Solid Bisphenol A (2,2-Bis(4-hydroxyphenyl) propane) with ≥ 99% purity (Sigma-Aldrich, molecular weight 228.291 g/mol) was dissolved in fish culture medium (E3 solution containing 5 mmol/L NaCl, 0.17 mmol/L KCl, 0.4 mmol/L $CaCl_2$, and 0.16 mmol/L $MgSO_4$) to prepare a 25 mg/L BPA stock solution. The tested BPA concentrations were then diluted in an E3 medium.

2.2. Zebrafish Maintenance and Pairing

The zebrafish (*Danio rerio*) AB strain was provided by the GIGA Research Center of the University of Liège (Belgium). Adult zebrafish were housed at 27 ± 1 °C with a 14-hour light: 10-hour dark photoperiod. They were fed twice daily with Tetramin flakes, Hikari Micro pellets, or boiled egg yolk. Fish water was dechlorinated tap water (pH 7-8). Our conditions follow Annex 2 of the 2013 OECD Standard 236 guidelines [25]. Zebrafish after one year old were selected for pairing with the male-to-female ratio usually 1:2 or 2:3 to maximize the spawn rate. Post-mating, embryos were collected into a Petri dish and washed multiple times with filtered water to eliminate debris. Fertilized embryos at the 4-8 cell stage, displaying no abnormalities, were selected under a microscope. Embryos were randomly gathered from at least three breeding pools to ensure sufficient sample size and minimize genetic bias.

2.3. Embryonic Exposure

BPA concentrations for the experiment were determined through the limit test according to OECD guidelines for testing of chemicals (OECD 236) [25]. The BPA concentration range of 0 (control), 2.5, 5, 10, 15, 20, and 25 mg/L was used. These were prepared by diluting the BPA stock solution (25 mg/L) with E3 medium and distributed into 6-well plates (4 mL/well). The fertilized embryos at the 4- to 8-cell stage were immediately selected and transferred to BPA-prepared wells (20 embryos/condition) and incubated at 23, 28, and 33 °C for 96 hours in Lovibond incubators. Half of the medium volume (2 mL) was changed; embryonic chorions and dead embryos were removed daily to minimize environmental contamination. Each condition was repeated at least three times. Data were recorded every 24 hours within 96 hours post-fertilization (hpf) using a stereo microscope, focusing on the number of dead, abnormal, and hatched embryos. Evaluation criteria for these outcomes were referenced from OECD guideline No. 236 [25] and Scopel et al., [26].

2.4. Data Analysis

Dose-response curves for the mortality and malformations of zebrafish embryos exposed to BPA and varying temperatures were plotted to determine LC_{50} and EC_{50} values. Differences in BPA toxicity across tests were assessed using Dunnett's multiple comparisons test in GraphPad Prism 8 and single-factor ANOVA (for lethality and malformation) and two-way ANOVA (for hatching rate) in Excel.

3. Results and Discussion

3.1. Effect of BPA and Temperature on the Mortality of Zebrafish Embryos

Zebrafish embryos were continuously exposed to BPA across a concentration gradient of 0, 2.5, 5, 10, 15, 20, and 25 mg/L under three different temperature conditions 23 °C, 28 °C (control), and 33 °C for 96 hours. Mortality rates at 24, 48, 72, and 96 hours post-fertilization (hpf) or hours of exposure (hrs) were presented as dose-response curves (Figure 1).

At 23 °C, almost no lethal effect of BPA was observed on zebrafish embryos throughout the testing period at the low concentrations (2.5 - 10 mg/L). Mortality witnessed a marked increase from 15 mg/L BPA at 72 hours. Higher concentrations and prolonged exposure intensified the effect, with mortality remaining below 20% at 24 and 48 hrs, however, a significant increase occurred in the last two days, reaching 100% by 96 hrs at 25 mg/L (Figure 1A).

Similar to the trend at 23 °C, at 28 °C, the lethal effects of BPA levels less than 15 mg/L were not detected at any observed time points (Figure 1B). However, a sharp increase in embryo mortality began at 15 mg/L from 72 hrs, and at 20 and 25 mg/L from 48 hours . Ultimately, all embryos exposed to 20 and 25 mg/L BPA perished by the end of the trial, with 100% mortality recorded over the final three days at 25 mg/L.

At 33 °C, the combined effect of elevated temperature and BPA exposure induced significant mortality, particularly at higher concentrations (Figure 1C). Mortality was evident across almost all exposure groups within the first 24 hours, except those exposed to the lowest concentration (2.5 mg/L). Interestingly, a contrasting lethality trend was observed at 5 mg/L and 10 mg/L of BPA; while 5 mg/L led to a significant decrease in viability only by 96 hours, mortality at 10 mg/L was evident from 24 hours and remained relatively stable throughout the trial. At higher concentrations (15 and 20 mg/L), less than 10% of embryos survived to 48 hours , and all embryos perished by 72 hours .

Across all temperature conditions, mortality rates generally increased in the rising of BPA concentration and exposure duration though the degree of toxicity varied among the different temperature settings (Figure 1A-C). It has been shown that the survival of embryos was not affected at the low dose (2.5 mg/L) of BPA in all temperature conditions. Compared to the

control (BPA - 28 °C), BPA at 33 °C significantly increased embryonic lethality across all concentrations, while at 23 °C, lethality appeared to be lower, especially at concentrations above 15 mg/L.

To assess the temperature-dependent toxicity of BPA on embryonic survival, LC_{50} values (the concentration of a test chemical that caused mortality for 50% of the test animals within a specified period) for different temperatures were derived from dose-response curves (Figures 1A-C) and summarized in Figure 1D. At 23 °C, LC_{50} values were only calculable from 72 hours and were higher than those at 28 °C and 33 °C. The lowest LC_{50} values were observed at 33 °C, particularly after 96 hours, where they were approximately 44% and 46% lower than at 23 °C and 28 °C, respectively, indicating the highest BPA toxicity at 33 °C. Furthermore, LC_{50} values tended to be decreased with longer exposure times, showing that higher temperatures and longer exposure increased BPA toxicity in zebrafish embryos ($F_{2,7}$ = 14.66, $p<0.05$). Our study determined that BPA at standard temperature (28 °C) had an acute effect on zebrafish embryos with LC_{50} values at 24, 48, 72, and 96 hours were 20.315, 20.751, 17.078, and 14.895 mg/L, respectively.

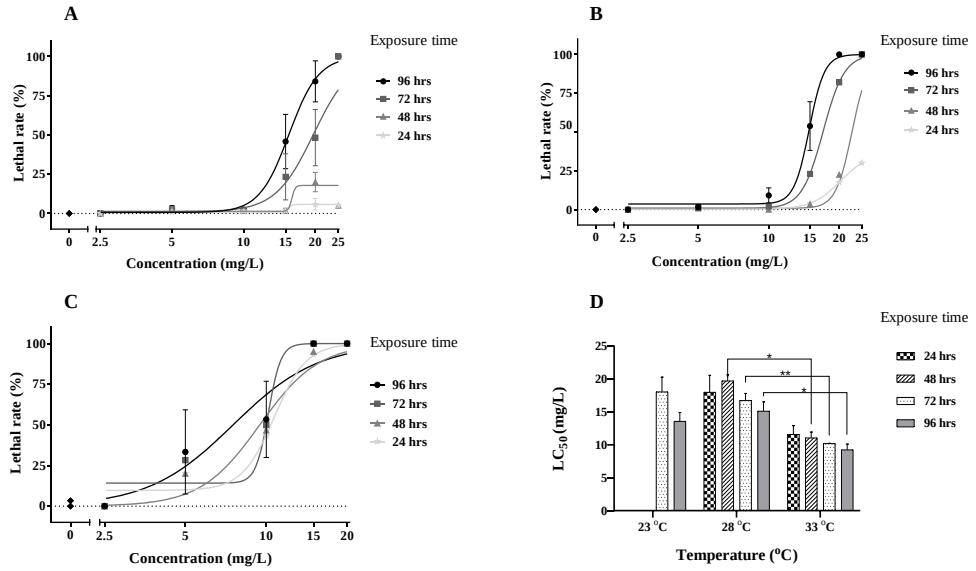

Figure 1. Effect of BPA on mortality of zebrafish embryos at 23 °C (A), 28 °C (B), 33 °C (C); data are expressed as mean (n=20) ± SEM. LC_{50} values (mg/L) at 24 – 96 hrs of exposure at different temperature conditions (D); data are expressed as mean ($n \geq 3$) ± SEM. Asterisks indicate statistical significance: "*" for $p < 0.05$; "**" for $p < 0.01$.

Compared with LC_{50} values of the previous report were 70 µM (15.980 mg/L), 72 µM (16.437 mg/L), 47 µM (10.730 mg/L), 31 µM (7.077 mg/L) given by Scopel et al., 2020 [26], the LC_{50} values observed in our study were higher at all time points but still at the similar trend and same order of magnitude. The difference between these results would be explained by the variance in the solvent used to dissolve BPA and the conditions of captivity among laboratories worldwide. Specifically, in the study of Scopel et al., [26], the solvent used was E3 solution containing 0.5% dimethylsulfoxide (DMSO) [26]. According to some studies on the toxicity of some common solvents such as Maes et al., [27], and Hallare et al., [28]; 0.5% DMSO was found to have no significant effect on the viability and normal development of zebrafish embryos within four days post-fertilization (dpf) [27, 28]. However,

it does not exclude the possibility that organic solvents may increase the solubility and delivery of toxic molecules to embryos [28].

3.2. Effect of BPA and Temperature on Zebrafish Embryo Morphology

Two primary malformations, hematoma (a small red clump inside the larvae), and pericardial edema, were observed in zebrafish embryos exposed to BPA at all temperature regimes. These defects, evident at different BPA concentrations and temperatures, are illustrated in Figure 2. These malformations are consistent with previous research on BPA toxicity in zebrafish embryos, suggesting a characteristic response to BPA exposure [29-31].

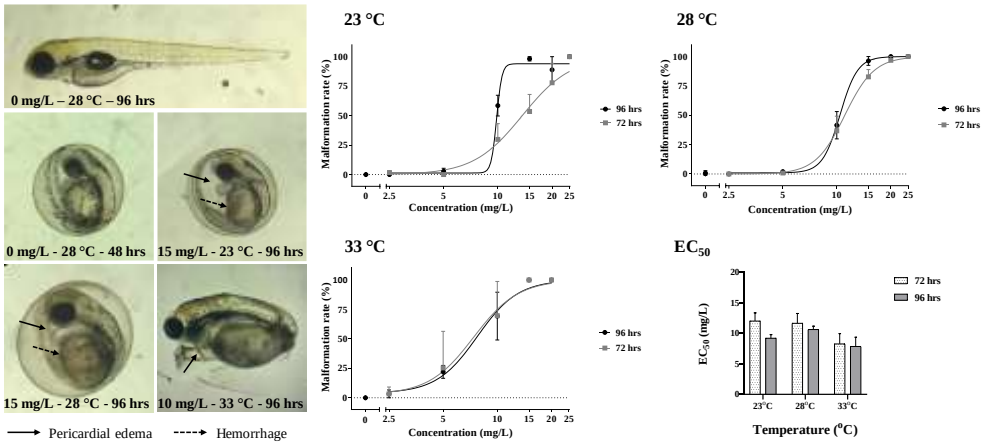

Figure 2. BPA-induced malformation in zebrafish embryos at 23 °C, 28 °C and 33 °C. Data are expressed as mean (n=20) ± SEM for dose-response curves and as mean (n ≥ 3) ± SEM for EC_{50} comparison.

Larvae exposed to BPA concentrations exceeding 10 mg/L exhibited paler complexion as opposed to individuals at 28 °C and 33 °C (data not shown), suggesting lesser melanin formation at the low temperature. Additionally, under closer inspection, the tail length of larvae at 20 mg/L of BPA was comparatively shorter than that of larvae at lower BPA concentrations. At 33 °C, deformation effects were detected as early as the 5 mg/L concentration, a pattern not observed under other conditions.

The rate of morphological malformation was calculated at 72 and 96 hours, as these abnormalities were observed during the last two days of the experiment. Consistent with mortality trends, BPA-induced malformations in zebrafish embryos increased with rising BPA concentrations and prolonged exposure time (Figure 2). At the two lowest BPA concentrations (2.5 and 5 mg/L), no morphological deformities were detected throughout the entire test period. However, at 10 mg/L, malformations were evident despite the absence of lethal effects. At concentrations of 15 mg/L or higher, nearly all surviving embryos exhibited one or more types of malformation, with the abnormality rate reaching 100% at 96 hours for 15 mg/L and 20 mg/L. Similar to the 23 °C condition, BPA - 28 °C also did not cause abnormalities in zebrafish embryos at BPA concentrations of 2.5 and 5 mg/L. At 10 mg/L, approximately half of the larvae were affected, and at 15 and 20 mg/L, all surviving larvae displayed one or more developmental defects. In alignment with the other temperatures, BPA toxicity at 33 °C increased with concentration and exposure time. However, the malformation effects were more pronounced, with deformities observed as early as 48 hours (data not shown) and at concentrations as low as 5 mg/L.

The EC_{50} is defined as the concentration of a test chemical that produces a specific biological effect in 50% of the experimental

subjects. In the context of this study, the EC_{50} value refers to the concentration that resulted in deformities in half of the surviving animals. It is important to note that if 100% mortality occurs, the abnormality rate is considered 100%. EC_{50} values differed between different temperatures but this difference was not statistically significant ($F_{2,3}$ = 3.60, p>0.05). Figure 2 displays that EC_{50} of BPA-33 °C is the lowest compared to the other two temperatures at both time points. Furthermore, the EC_{50} values at 23 °C and 28 °C exhibited a reduction with prolonged exposure time. The EC_{50} values at 72 hours were higher than those observed at 96 hrs. However, at 33 °C, the EC_{50} values at both 72 and 96 hrs were relatively comparable, measuring 7.380 mg/L and 7.731 mg/L, respectively. This phenomenon could be explained by the fact that some embryos carrying malformations were alive at 72 hours but died right the next day (at 96 hours of exposure).

3.3. Effect of BPA and Temperature on the Hatching Rate of Zebrafish Embryos

Figure 3 shows that there was a combined impact of temperature and BPA on the embryonic hatching of zebrafish embryos. At 48 hrs, no hatched embryo was recorded at 23 °C (Figure 3A). The percentage of hatched embryos increased at higher temperatures at concentrations below 10 mg/L, which was supported by the ANOVA result. Notably, at the control condition, the hatching rate of embryos at 33 °C was 25-fold higher than that at 28 °C. These results suggest that the temperature elevation stimulated the hatching of embryos. However, the hatching at 33 °C decreased in the presence of BPA.

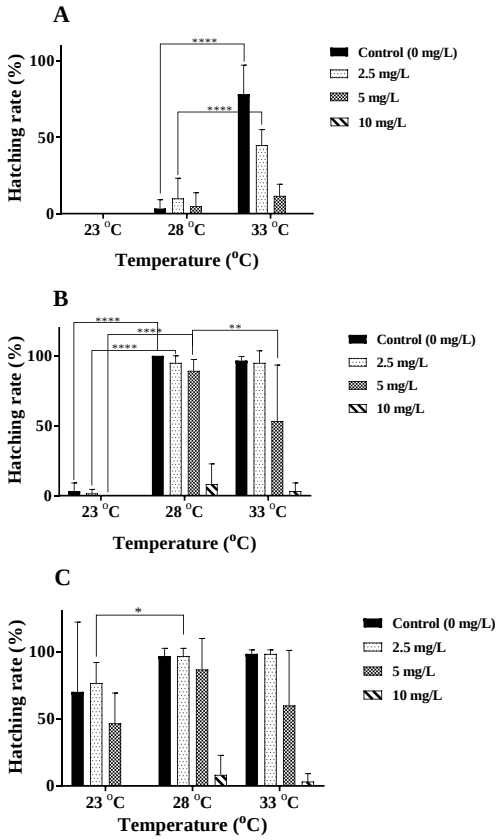

Figure 3. Hatching rate (%) of zebrafish embryos after exposure to BPA and temperature; at 48 hrs (A), 72 hours (B), and 96 hours (C). Data are expressed as mean (n=20) ± SD. Asterisks indicate statistical significance: '*' for p < 0.05; '**' for p < 0.01; '***' p < 0.001; '****' for p < 0.0001).

4. Conclusion

In this study, Bisphenol A showed the toxic effect on zebrafish (*Danio rerio*) embryonic development at concentrations higher than 10 mg/L, leading to increasing of malformation and mortality rate, and delaying hatching. Moreover, the toxicity of BPA was enhanced by increasing temperature and exposure duration, suggesting the combined influence of these abiotic factors on the embryonic development of zebrafish.

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