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Synergistic Developmental Toxicity and Apoptosis of BPA and Zn Co-exposure in *Daphnia magna*

(Ketoksikan Perkembangan Sinergi dan Apoptosis Pendedahan BPA dan Zn CO dalam *Daphnia magna*)

NAIMA HAMID[1,2,*], AISHAH BINTI ARIFIN[1] & ONG MENG CHUAN[1,2]

[1]*Faculty of Science and Marine Environment, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia*
[2]*Ocean Pollution and Ecotoxicology (OPEC) Research Group, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia*

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ABSTRACT

The combined effects of chemical pollutants in the aqueous environment create inevitable impacts on aquatic ecosystems. Bisphenol A (BPA) is identified as a chemical pollutant of significant concern, primarily owing to its pronounced toxicity in the freshwater environment. Similarly, essential elements like zinc (Zn) may induce toxic effects upon alterations in their metal speciation, leading to increased bioavailability. Therefore, the present study aimed to elucidate the developmental toxicity effects using fresh water model *Daphnia magna*. Using environment-relevant BPA (10 µg/L and 20 µg/L) and Zn (10 µg/L and 30 µg/L) levels were exposed to *Daphnia magna* for 48 h to determine the acute toxicity. Results showed that maximum mortality (40%) was observed at a high exposure dose of BPA and Zn at 48 h. Similarly, a significant dose-dependent reduction in heartbeat and body weight was found in all samples. Blood clotting, broken antenna, missing tail changes in the carapace, and bioaccumulation, were the dominant morphological changes in all treatments. These findings were confirmed with the elevated apoptosis at the second antenna, gut, and post-abdominal regions after high-dose treatment. Spearman correlation analysis showed a significant positive correlation ($R^2 = 0.86$) between mortality and deformity ratio indicating high joint toxicity of BPA and Zn. Lastly, more in-depth studies are required to highlight the combined toxicity of the BPA and Zn and ensure ecological integrity.

Keywords: Acute toxicity; Bisphenol A; *Daphnia magna*; emerging pollutants; heavy metals

ABSTRAK

Kesan gabungan pencemar kimia dalam persekitaran akuatik memberi kesan yang tidak dapat dielakkan terhadap ekosistem akuatik. Bisfenol A (BPA) dikenal pasti sebagai pencemar kimia yang memberi perhatian utama, terutamanya disebabkan ketoksikan yang ketara dalam persekitaran air tawar. Demikian juga, unsur-unsur penting seperti zink (Zn) boleh menyebabkan kesan toksik apabila terdapat perubahan dalam spesiasi logam mereka, mengakibatkan peningkatan bioketersediaan. Oleh itu, kajian ini bertujuan untuk menerangkan kesan ketoksikan perkembangan dengan menggunakan model air tawar *Daphnia magna.* Dengan menggunakan tahap BPA (10 µg/L dan 20 µg/L) dan Zn (10 µg/L dan 30 µg/L) yang relevan dengan alam sekitar, *Daphnia magna* terdedah selama 48 jam untuk menentukan ketoksikan akut. Keputusan menunjukkan bahawa kematian maksimum (40%) diperhatikan pada dos pendedahan tinggi BPA dan Zn pada 48 jam. Begitu juga, pengurangan berdasarkan dos yang signifikan dalam kadar degupan jantung dan berat badan ditemui dalam semua sampel. Pengerutan darah, patah antena, perubahan ekor yang hilang dalam karapas, dan bioakumulasi, adalah perubahan morfologi dominan dalam semua rawatan. Penemuan ini disahkan dengan peningkatan apoptosis pada antena kedua, usus dan kawasan post-abdomen selepas rawatan dos tinggi. Analisis korelasi Spearman mendedahkan korelasi positif yang signifikan ($R^2 = 0.86$) antara kadar kematian dan nisbah kecacatan, menunjukkan ketoksikan gabungan yang tinggi bagi BPA dan Zn. Kesimpulannya, kajian yang lebih mendalam diperlukan untuk menonjolkan ketoksikan gabungan BPA dan Zn serta memastikan integriti ekologi.

Kata kunci: Bisfenol A; *Daphnia magna*; kemunculan bahan pencemar; ketoksikan akut; logam berat

Introduction

In recent times, there has been substantial global attention directed towards bisphenol A (BPA) due to its persistence in the environment and the consequential toxicological effects (Zhang et al. 2023). BPA is a chemical compound belonging to the diphenylmethane derivative class. It has been utilized for several decades in the manufacturing of polycarbonate plastics, epoxy resins, and a diverse range of consumer products based on plastic materials (Metruccio et al. 2024). The heightened interest in BPA is attributed to the exceptional thermal and photolytic stability, as well as resistance to microbial degradation, conferred by its high-energy carbon-fluorine (C-F) bond resulting high environmental distribution and bioaccumulation (Wang et al. 2021). Currently, given the rising frequency and concentration of detection of BPA in environmental media (Liang et al. 2023), there is a pressing need for further investigation into its ecological safety.

Among the analogues of BPA, bisphenol B (BPB) is utilized as an alternative in plastic production in various non-EU countries, including the United States (Metruccio et al. 2024). Despite regulatory restrictions, BPA continues to be used in various industries and prevalent in surface water (Kundu et al. 2024), sediments (Liu et al. 2021), and biota (Liang et al. 2023). Bisphenol A (BPA) is categorized among endocrine disruptors due to its capacity to mimic estrogen binding and display anti-androgenic activity. Recognizing its potential adverse effects, the European Chemicals Agency (ECHA) has designated BPA as a 'substance of very high concern' (Chen et al. 2019). Previously, toxicological studies on BPA have predominantly focused on its potential impact on endocrine disruption, immune system disorders, neurotoxicity, and reproductive developmental effects (Chen et al. 2017; Scopel et al. 2020; Wang et al. 2021). Early life stages of both invertebrates and vertebrates exhibit high sensitivity to BPA exposure, with invertebrates and amphibians appearing to be particularly susceptible to its effects (Liu et al. 2021). However, research focused on environmentally relevant exposure to BPA remains limited and elusive.

In a study conducted by Liu et al. (2021), the high acute toxicity of BPA was identified, with an EC_{50} of 19.6 mg/L, followed by Bisphenol S (BPS) with an EC_{50} of 361 mg/L (Liu et al. 2021). Similarly, in a study exploring the acute toxicity of BPA across various trophic levels, including microalga (*Tetraselmis* sp.), zooplanktonic grazer (*Artemia salina*), deposit-feeder invertebrate (*Heleobia australis*), and omnivorous fish (*Poecilia vivipara*) (Naveira et al. 2021). *Tetraselmis* sp. demonstrated the highest tolerance to BPA, exhibiting a non-concentration-dependent response (Naveira et al. 2021). Migloili et al. (2021) demonstrated the developmental toxicity and genetic expression alteration in marine bivalve *Mytilus galloprovincialis* after exposure to 0.05-5 μM BPA at 48 hours post-fertilization (Hpf). The exposure to BPA led to impairment in the development of serotonin-5-HT-immunoreactive neurons, contributing to developmental delays in the marine bivalve. Similarly, BPA induced morphological and physiological alterations in zebrafish, including yolk sac and pericardial edema, hatching delay or inhibition, spine deformation, a decrease in heartbeat rate, and increased mortality when exposed to 25 μM BPA after 96 hpf (Chen et al. 2019).

Combined toxicity assessment is a realistic yet challenging approach to evaluating chemical interactions and the associated health risks. However, there are no studies available about the combined toxicity of BPA and Zn on aquatic species. It is noteworthy that essential elements, including zinc (Zn), may induce toxic effects when alterations in their metal speciation occur, making them bioavailable (Price et al. 2023). Previously, it was reported that dissolved organic matter (DOM) jointly exposed to Zn caused reproductive toxicity in *Daphnia magna* (Heijerick, Janssen & De Coen 2003). Furthermore, Hyne et al. (2005) demonstrated acute developmental toxicity in *Ceriodaphnia dubia* following exposure to 10 mg/L of zinc (Zn). More recently, Zhan et al. (2023) illustrated the chronic (30 days) potential for Zn toxicity on Drosophila melanogaster and its trophic transfer to *Pardosa laura* after exposure to concentrations of 0.25 mg/mL and 0.50 mg/mL.

The zooplankton *Daphnia magna* is a frequently employed freshwater model in ecotoxicological testing, owing to its ease of maintenance, transparency, and high fecundity, as highlighted by Labine et al. (2023). Measuring only 2-5 mm in length, Daphnia magna possesses the characteristic shape of a kidney bean (Houde et al. 2016). Beyond its role in ecotoxicological studies, Daphnia magna is ecologically significant and serves as a bioindicator species, widely distributed across freshwater aquatic ecosystems. Therefore, the present study aims to determine the acute toxicity potential of BPA and Zn exposed at environmentally relevant concentrations (ERC). The assessment includes the identification of apoptosis using acridine orange (AO) staining after chemical exposure.

Materials and Methods

Ethics statement

Daphnia magna experiments were carried out in accordance with protocols approved by the Animal Care and Use Committee of the University Malaysia Terengganu (UMT), Malaysia. (Approval ID: UMT/JKEPHMK/2023/107).

CHEMICALS REAGENTS AND EXPERIMENTAL DESIGN

All chemicals were acquired from AccuStandard with a purity of 99.6% (New Haven, CT, USA). Bisphenol A (BPA) with CAS number 80-05-7 and Zinc sulfate (ZnSO4) with CAS number 7733-02-0 were utilized in the study. BPA and ZnSO4 were dissolved separately in dimethyl sulfoxide (DMSO) and UltraPure water (ddH2O) to create a stock solution with a concentration of 1 mg/L. Subsequently, the stock solution was further diluted using UltraPure water (ddH2O) with a resistance of 18.2 MΩ to achieve the desired concentrations of BPA (10 µg/L and 20 µg/L) and Zn (10 µg/L and 30 µg/L). The chemicals (BPA and Zn) were concurrently administered to Daphnia magna at environmentally relevant concentrations (ERC). For each treatment, a total of N = 30 individuals were utilized, with codes assigned to represent control, low-dose, and high-dose groups (C1, C2, C3, LD1, LD2, LD3, HD1, HD2, HD3), respectively.

Daphnia magna HUSBANDRY, ACUTE EXPOSURE, AND SAMPLE COLLECTION

Daphnia magna were cultured and maintained in a flow-through system within the hatchery department at the Universiti Malaysia Terengganu, under controlled conditions of 28 ± 0.5 °C and a 14:10 light–dark cycle. Green algae were provided to the infants as food twice daily. Healthy adult Daphnia magna individuals were transferred into 500 mL beakers containing 300 mL of a combined solution of BPA and Zn, each at their respective concentrations, for a 24-h exposure period. The experimental setup adhered to the guidelines outlined in OECD Test No. 211 (OECD 2012). The exposed solution was replaced daily with a freshly prepared stock solution to maintain the designated final mixture concentration. Each treatment was independently replicated three times with distinct biological replicates. Subsequent to the 24-h exposure, all Daphnia from both the control and treated groups were preserved at -20 °C for AO staining.

DEVELOPMENTAL TOXICITY

Throughout the exposure period, various developmental toxicity parameters, including mortality, heartbeat, body weight, and deformities, were systematically monitored at 12-h intervals. Observations on the heartbeat rate and deformities of *Daphnia magna* were conducted using an inverted microscope every 12 h. Given the rapid heartbeat rate of *D. magna*, a stopwatch was employed to record the number of heartbeats per minute. Morphological abnormalities, encompassing changes in body structure, antenna length, tail deformation, and carapace disruption, were also scrutinized for each observation interval. This comprehensive assessment aimed to capture any potential adverse effects on developmental processes and overall physiological health of D. magna under the influence of the combined BPA and Zn exposure.

ACRIDINE ORANGE (AO) STAINING

Acridine Orange (AO) staining, is a metachromatic stain selective for nucleic acids and commonly employed to examine apoptosis patterns (Kari et al. 2022). It was utilized to identify apoptosis *in vivo* within *Daphnia magna* exposed to a combined BPA and Zn mixture. The staining procedure followed the method outlined by Hamid et al. (2020). Initially, an AO stock solution was prepared and homogeneously mixed to achieve a final concentration of 100 g/mL. Subsequently, *Daphnia magna* were immersed in the AO solution for a 30-min incubation period at 28 °C. Following the staining process, *D. magna* underwent four successive washes with 1 × PBS, maintaining a normalized pH level of 7.4. The stained samples were meticulously pipetted onto glass slides for observation of apoptosis using an inverted fluorescence microscope with a 10× CLSM (Confocal Laser Scanning Microscope). This approach allowed for the visualization and analysis of apoptosis patterns within *D. magna* exposed to the joint BPA and Zn mixture, providing valuable insights into potential cellular responses and impacts on apoptotic pathways in the studied organisms.

DATA ANALYSIS

The normality of the data was assessed through the application of the Kolmogorov-Smirnov test. Subsequently, the developmental indices data were subjected to statistical analysis using GraphPad Prism 10 version, employing a two-way analysis of variance (ANOVA). A significance level of $P < 0.05$ was established to identify statistically significant differences. Spearman correlation analyses were conducted using the Heat Mapper online software available at http://www.heatmapper.ca/pairwise/.

RESULTS AND DISCUSSION

COMBINED BPA AND ZN EXPOSURE CAUSED DEVELOPMENTAL TOXICITY IN *Daphnia magna*

Mortality

In this study, joint exposure to BPA + Zn at ERCs induced significant developmental toxicity following acute exposure for 48 h. In the low-dose treatment group, the highest mortality rates were observed at 48 h (27.7%), 36 h (18.8%), 24 h (12.2%), and 12 h (8.8%), as depicted in Figure 1(A). Similarly, in the high-dose treatment group, the maximum mortality occurred at 48 h (40.0%), followed by 36 h (25.5%), 24 h (18.8%), and 12 h (13.3%), respectively. Previously, Zhang et al. (2023)

reported that at the high concentration group (200 µg/L), the survival rate of *Daphnia magna* noticeably decreased with the increase in exposure generations. Naverira et al. (2021) highlighted an elevated mortality rate among BPA concentrations (300 mg L^{-1}) from 48 to 96 h of exposure in *Artemia salina*.

Heartbeat

Changes in the heartbeat of Daphnia magna were also observed at 12-h intervals of exposure to BPA + Zn (Figure 1(B)). The results indicated a dose-dependent decrease in heartbeat across all groups, except in the high-dose (HD) group compared to the control. In the low-dose group, the maximum heartbeat was reduced to 400 beats/min at the 48-h time interval. Similarly, a lower heartbeat of 260 beats/min was observed after high-dose exposure at 36 h. These findings align with a previous study that highlighted a reduction in heartbeats with higher doses of BPA in *Daphnia magna* (Liang et al. 2017). According to Liu et al. (2020), heart rate (HR) has been commonly employed to assess the stress levels in *Daphnia magna* exposed to BPA, as it closely correlates with respiratory, feeding, and metabolic functions.

Growth rate

The growth rate, indicative of body weight, serves as a crucial parameter for monitoring the developmental toxicity of chemicals. Exposure to BPA and Zn resulted in a significant decrease in body weight across all treated groups. In high-exposure scenarios, a dominant dose-dependent trend was observed, with a maximum decrease of 0.003 g followed by 0.007 g after 48 h of exposure, compared to the control. Similar trends were found for low-dose exposure at 12 h, 36 h, and 48 h, with body weights of 0.012 g, 0.008 g, and 0.002 g, respectively, except at 24 h, where the initial weight of 0.017 g was recorded (Figure 1(C)). Similarly, Seyoum et al. (2020), reported that the body weight and length of *Daphnia magna* were reduced significantly by 25 µM PFOS exposure for 7 days. Similar findings were observed in previous studies on BPA toxicity, wherein prolonged generational exposure to 1140 µg/L of BPA significantly reduced the body size of offspring *Daphnia magna* (Chen et al. 2021). However, the inhibitory effect of phenolic compounds on the levels of thyroid hormones (T3 and T4) may be the primary cause of growth retardation and reduction in body weight and body length observed in *Daphnia magna* (Zhang et al. 2023).

Deformity rate

Short-term co-exposure to BPA and Zn resulted in noticeable morphological alterations in Daphnia magna across all treatment groups. In the low-dose treatment, a significant rise in deformities was observed in a dose-dependent manner, as compared to the control (Figure 1(D)). The overall trend for the low dose exhibited an increase in the order of 12.2% < 17.7% < 24.4% < 31.1% at 12 h, 24 h, 36 h, and 48 h of exposure. Similarly, for the high dose, the deformity ratios escalated with percentages of 15.5%, 22.2%, 32.2%, and 40.0% at the corresponding time intervals. A low concentration of bisphenol S (BPS) significantly inhibited the heart rate (HR) and swimming activity of *Daphnia magna* across generations (F0–F3). (Zhang et al. 2023). Li et al. (2018) demonstrated that chronic exposure of *Daphnia magna* to BPA and its metabolites can lead to an insufficient supply of reproductive energy and hinder its reproductive function.

MORPHOLOGICAL DEFECTS OF COMBINED BPA + ZN IN *Daphnia magna*

Acute exposure of combined PFOA and Zn has caused serious morphological changes in all treatments (Figure 2(A) and 2(B)). The obvious phenotypes were BT- Broken Tail, BC- Blood Clotting, CHS- Changes in Head Shape, BA- Broken Antenna, MT- Missing Tail, SBC- Shrinking Brood Chamber, CC- Changes in Carapace, ME- Moved Embryo, MA- Missing Antenna, BA- Bioaccumulation, OT- Overgrowth Tail, BI- Bent Intestine, MCE- Missing Compound Eye and SO- Shrinking Organ in *D. magna*. In low-dose samples, the majority of the daphnia experienced BI, MT, BC, MCE, CC, and SO, compared to the control (Figure 2(A)). Results were similar after investigating the toxicological effects of ibuprofen and silver nanowires (Grzesiuk et al. 2020; Park et al. 2021). Likewise, MT, MA, BA, BT, and OT were found after 36 h and 48 h of BPA and Zn joint exposure (Figure 2(B)). Teratogenic effects identified in living tadpoles included anterior defects such as a round head, shortened and reduced gill basket and ventral edema following exposure to *Xenopus laevis* species at levels ranging from 0 to 35 µM (Metruccio et al. 2024). It can be inferred that the joint exposure to BPA and Zn resulted in an elevation of developmental toxicity in *Daphnia magna*. In addition, bioaccumulation at 36 h and 48 h might be responsible for serious metabolic and reproductive effects. Xie et al. (2019) also confirms that with the increased exposure time, aquatic organisms with short breeding cycles become particularly vulnerable to the dual pressure of direct exposure toxicity and increase the genotoxic effects.

INCREASED APOPTOSIS LEVELS

Following joint exposure to the mixture of BPA and Zn, the acridine orange (AO) stained Daphnia magna was observed under a green stereomicroscope (Figure 3). Primarily, LD-treated samples exhibited increased apoptosis in the first thoracic appendage region and the

heart, both at the dorsal and ventral sides, compared to the control (Figure 3(A) and 3(B)). Similarly, Figure 3(C) illustrated elevated apoptosis at the second antenna, gut, and post-abdominal regions after HD treatment with the joint exposure of BPA and Zn. Comparable results were observed when *Daphnia magna* were jointly exposed to PFOA and PFOS, displaying high bioaccumulation as indicated by Oil Red O staining (Seyoum et al. 2020). Previously, it was confirmed that BPA induces apoptosis through oxidative stress in various cellular models (Metruccio et al. 2024). The literature has demonstrated that BPA exhibits not only acute toxicity and high cytotoxicity but also the potential for genotoxic effects (Usman, Ikhlas & Ahmad 2019). In a study conducted by Wang et al. (2021), it was observed that BPA induced oxidative stress and apoptosis. Interestingly, the effect of co-exposure to BPA and SiO_2 nanoparticles was found to be less pronounced compared to the impact of single exposure, particularly at a concentration of 10 µg/mL. The trend in fluorescence intensity also aligned with the staining results, indicating dose-dependent toxicity of the combined exposure to BPA and Zn in *Daphnia magna.*

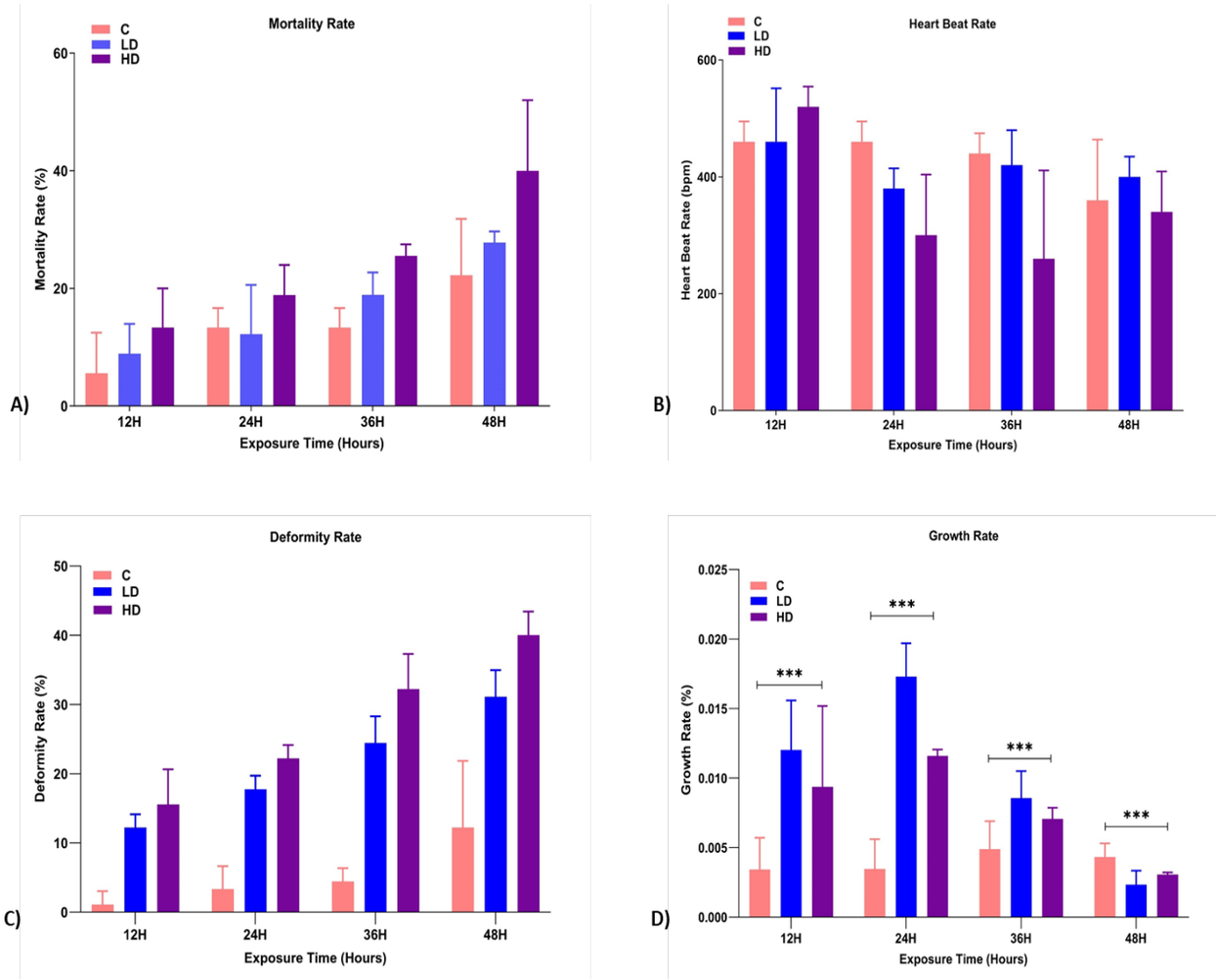

FIGURE 1. Graphs showing the joint BPA and Zn mixture exposed to *D. magna* for 48 h after fertilization. Following acute exposure, developmental lethal (mortality rate and deformity ratio) and non-lethal (heartbeat rate and body weight) malformations were observed at environmental relevant concentrations (ERCs). The data presented are three replicate groups. Two-way ANOVA significance level: *p < 0.05, **p < 0.01, ***p < 0.001

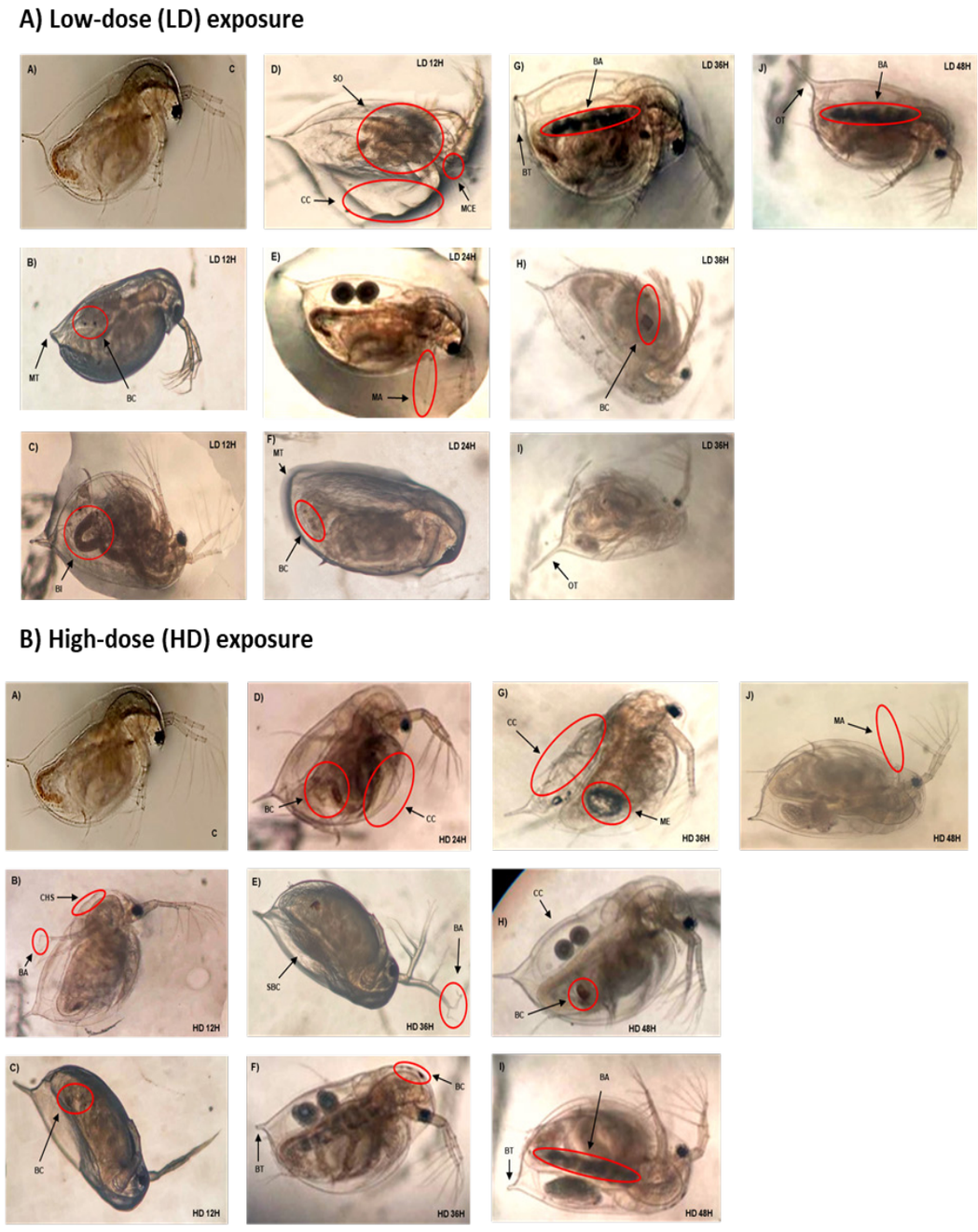

FIGURE 2. Malformations of *D. magna* caused by acute exposure (low dose and high dose) to BPA and Zn at ERCs. *D. magna* toxicological endpoints showed morphological malformations at 12 h, 24 h, 36 h, and 48 h hpf. BT- Broken Tail, BC- Blood Clotting, CHS- Changes in Head Shape, BA- Broken Antenna, MT- Missing Tail, SBC- Shrinking Brood Chamber, CC- Changes in Carapace, ME- Moved Embryo, MA- Missing Antenna, BA- Bioaccumulation, OT- Overgrowth Tail, BI- Bent Intestine, MCE- Missing Compound Eye and SO- Shrinking Organ. LD: low dose, HD: high dose, C: Control. Scale: 400 × magnification

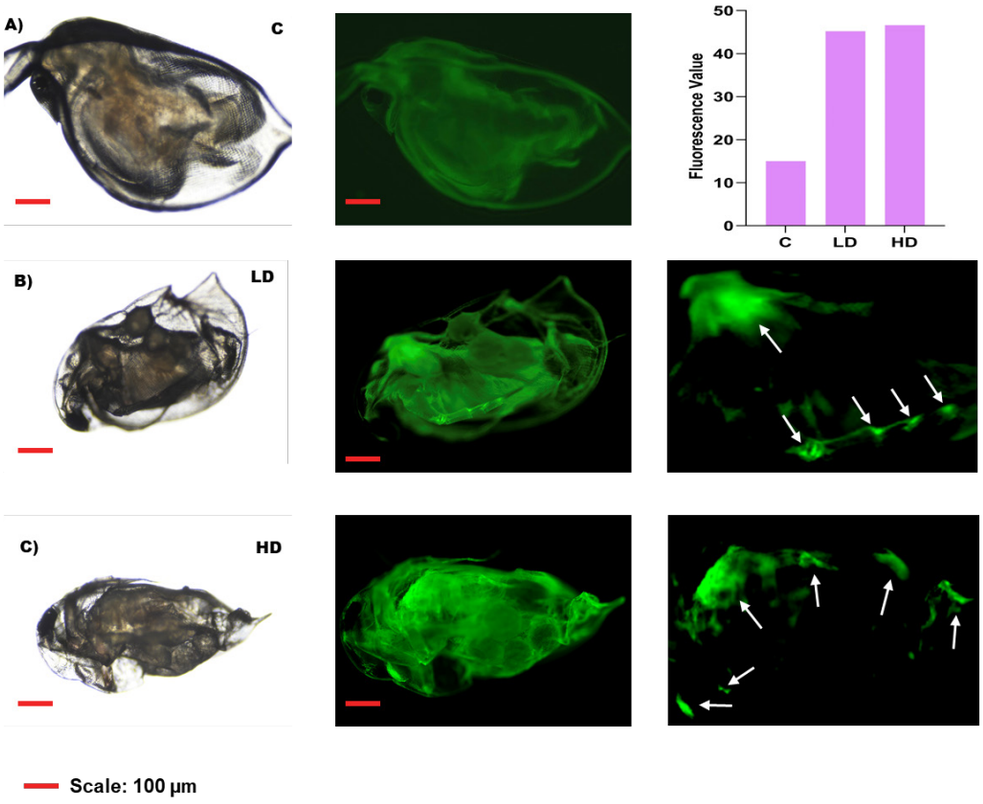

FIGURE 3. Acridine orange staining shows apoptosis with green fluorescence following joint exposure of BPA and Zn at ERC concentration. Photographs were captured on the bright field and dark field with a scale of 100 µm. (F) Fluorescence intensity values of apoptosis were calculated using Image J

SPEARMAN CORRELATION

Spearman correlation analysis was performed to elucidate the relationship among developmental toxicity parameters (Figure 4). Results showed that mortality and deformity ratio were significantly positively correlated with the correlation coefficient $R^2 = 0.86$ among all samples, compared to control. Contrarily, both heartbeat rate and deformities indicated a negative association with the $R^2 = -0.63$. Similarly, growth rate vs mortality ($R^2 = -0.38$), and heartbeat rate vs growth rate ($R^2 = -0.06$) were negatively correlated in all samples (Table 1). These findings further statistically validated the experimental results, indicating the higher toxicity potential of PFOA and Zn in *Daphnia magna*. Previous study also showed a positive correlation between BPA-related teratogenic effects and apoptosis (Metruccio et al. 2024).

TABLE 1. Spearman correlation results show the association between developmental toxicity parameters observed in *D. magna*

Parameters	Mortality rate	Growth rate	Heartbeat rate
Growth rate	-0.38	1	-
Heartbeat rate	-0.61	-0.06	1
Deformity rate	**0.86**	0.004	-0.63

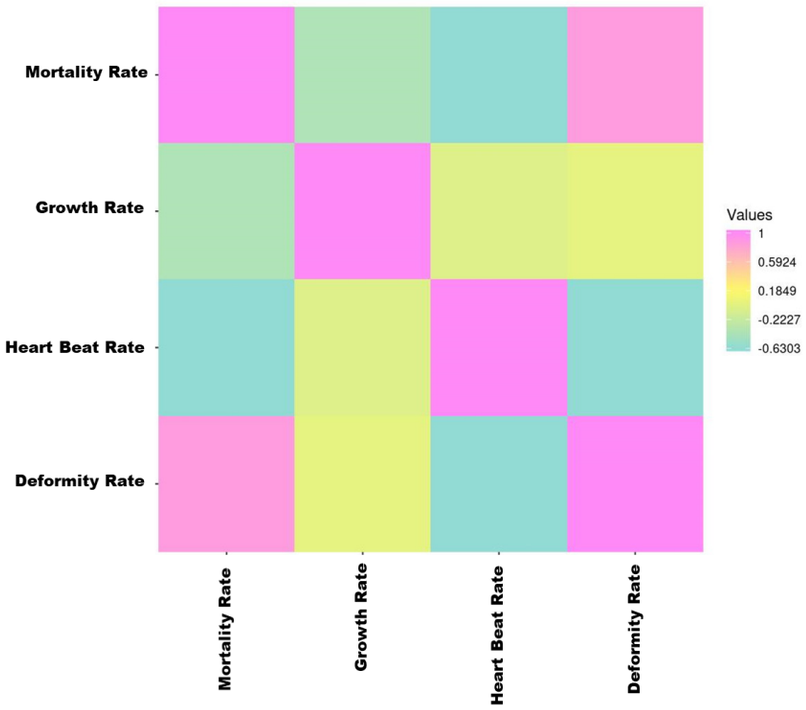

FIGURE 4. Heat maps depicting the Spearman rank correlation of developmental toxicity parameters (mortality, body weight, deformities ratio, and heartbeat rate) observed 48 h after treatment. Significance levels: *p < 0.05. Rainbow color gradient showing the positive correlation coefficient from yellow to pink color

CONCLUSIONS

The present study was conducted to elucidate developmental toxicity and identify apoptosis using the freshwater model *Daphnia magna* and confirm its toxicity potential through *in silico* molecular docking. *Daphnia magna* were exposed to environmentally relevant levels of BPA and Zn for 48 h to assess acute toxicity. Results indicated a higher mortality at 40% maximum at a high exposure dose for 48 h. Furthermore, a notable dose-dependent reduction in heartbeat and body weight was observed across all samples. Morphological changes such as blood clotting, broken antennae, missing tails, carapace alterations, bioaccumulation, and a shrinking brood chamber were predominant in all treatments. High-dose treatment resulted in elevated apoptosis at the second antenna, gut, and post-abdominal regions. Spearman correlation analysis showed a significant positive correlation (R^2 = 0.86) between mortality and deformity ratio, indicating a substantial joint toxicity of BPA and Zn. In summary, more in-depth studies are required to unveil the mechanistic toxicity of joint BPA and Zn in the aquatic environment (Supplementary S1-S4).

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*Corresponding author; email: naima@umt.edu.my

TABLE S1. Summarize mortality rate data for the acute developmental toxicity in *Daphnia magna*

Mortality rate (%)									
Exposure time	Control			Low dose			High dose		
12 h	0	13.33	3.33	10	3.33	13.33	6.67	13.33	20
24 h	10	16.67	13.33	13.33	3.33	20	13.33	20	23.33
36 h	10	16.67	13.33	16.67	16.67	23.33	23.33	26.67	26.67
48 h	16.67	33.33	16.67	26.67	30	26.67	36.67	53.33	30

TABLE S2. Summarize heartbeat rate data for the acute developmental toxicity in *Daphnia magna*

Heartbeat rate (bpm)									
Exposure time	Control			Low dose			High dose		
12 h	480	480	420	360	480	540	480	540	540
24 h	480	480	420	420	360	360	180	360	360
36 h	420	480	420	480	420	360	120	240	420
48 h	300	300	480	360	420	420	420	300	300

TABLE S3. Summarize deformity rate data for the acute developmental toxicity in *Daphnia magna*

Deformity rate (%)									
Exposure time	Control			Low dose			High dose		
12 h	0	0	3.33	10	13.33	13.33	16.67	20	10
24 h	6.67	0	3.33	16.67	20	16.67	23.33	23.33	20
36 h	6.67	3.33	3.33	20	26.67	26.67	33.33	36.67	26.67
48 h	23.33	6.67	6.67	26.67	33.33	33.33	40	43.44	36.67

TABLE S4. Summarize growth rate data for the acute developmental toxicity in *Daphnia magna*

Growth rate (%)									
Exposure time	Control			Low dose			High dose		
12 h	0.0014	0.003	0.0059	0.0086	0.0157	0.0118	0.0041	0.0084	0.0156
24 h	0.003	0.0016	0.0058	0.0197	0.0173	0.0149	0.0117	0.012	0.0111
36 h	0.0026	0.0063	0.0058	0.0108	0.0074	0.0075	0.008	0.0066	0.0066
48 h	0.0035	0.0054	0.0041	0.0018	0.0035	0.0017	0.0029	0.0031	0.0032