American Journal of Environmental Sciences

Research Article

The interplay between Cadmium and Zinc Induced Developmental Perturbations and Apoptosis in Daphnia Magna

[1,2]Naima Hamid, [1]Siti Nur Airina Binti Ibrahim, [3,4]Muhammad Junaid and [5]De-Sheng Pei

[1]*Faculty of Science and Marine Environment, University Malaysia Terengganu, Kuala Nerus, Terengganu, Malaysia*
[2]*Ocean Pollution and Ecotoxicology (OPEC) Research Group, University Malaysia Terengganu, Malaysia*
[3]*College of Marine Sciences, South China Agricultural University, Guangzhou, China*
[4]*Guangdong Provincial Key Laboratory of Utilization and Conservation of Food and Medicinal Resources in Northern Region, Shaoguan University, Shaoguan, China*
[5]*School of Public Health, Chongqing Medical University, Chongqing, China*

Article history
Received: 25-06-2024
Revised: 15-07-2024
Accepted: 24-07-2024

Corresponding Author:
De-Sheng Pei
School of Public Health, Chongqing Medical University, Chongqing, China
Email: peids@cigit.ac.cn

Abstract: Assessing combined toxicity provides a realistic and comprehensive approach to determining toxicological effects in aquatic species under environmentally relevant conditions. This study was conducted to understand the effects of single and combined metal mixtures on freshwater flea and *Daphnia magna*. Environmentally Relevant Concentrations (ERCs) of Cadmium (Cd) and Zinc (Zn) at a low dose (10 µg/L) and high dose (30 µg/L) were employed for single and joint acute exposure (48 h) to investigate the associated mortality rate, heartbeat rate, growth rate, deformities rate, morphological changes and Apoptosis. Compared to a single exposure, the combined exposure of Cd and Zn induced elevated developmental toxicity, with pronounced bioaccumulation in the gut. Further, co-exposure showed the highest mortality (62%) in a dose-dependent treatment, compared to single-exposure groups. In all the treatments, the predominant morphological defects included missing tail, antenna, bioaccumulation, blood clotting, carapace alterations and shrinking organs, compared to the control group. However, faster bioaccumulation was observed after 12 h of Cd + Zn co-exposure. Furthermore, Acridine Orange (AO) staining revealed high Apoptosis, specifically in the head, antennae and gut or abdominal area in all samples. The Spearman correlation indicated a significant positive correlation between deformity and mortality rate in *D. magna*, suggesting the synergistic toxicity of Cd + Zn. In conclusion, single and mixture exposure to Cd + Zn at the ERC induced developmental impacts and Apoptosis in *D. magna*. Here, the combined toxicity assessment of heavy and trace metals mimics the realistic scenario, aiding authorities in further regulating the use of these elements and minimizing associated ecological health risks.

Keywords: Metals Toxicity, *in vivo* Impacts, *Daphnia magna*, Acute Exposure, Apoptosis

Introduction

In recent decades, the combined toxicity of heavy metals has emerged as a significant environmental challenge that cannot be overlooked. Trace elements such as Cadmium (Cd) and Zinc (Zn) are pivotal in various industrial processes and biological functions Cukrov *et al.* (2008). They are used as a by-product in various processes, such as the mining and refining of zinc sulfide ores, as well as coal and the electroplating industry (Garcia-Santos *et al.*, 2015). These elements are introduced into aquatic ecosystems through direct or indirect waste disposal, causing significant health impacts on aquatic species Gong *et al.* (2021).

In freshwater environments, the concentration of total dissolved cadmium typically remains below 0.5 µg L^{-1} (Garcia-Santos *et al.*, 2015). However, previous studies found elevated levels of Cd (0.7 mg/L) and Zn (2.79 mg/L) in Milliardaires Bay, France (Pan *et al.*, 2010). The concentration of Cd ranged between 3-8 ng/L in the Krka River, Croatia (Cukrov *et al.*, 2008). In Northern

England, Zn concentration was measured at 0.216 mg/L in the Derwent reservoir (Harding & Whitton, 1978). Similarly, studies reported zinc levels (20 μg/L) in the Mississippi River, respectively, in the United States (Shephard *et al.*, 1980). The fingerprints of Cd and Zn in surface water, along with their bioaccumulation in the food chain, are hazardous to aquatic species.

Bioaccumulation of Cd leads to severe developmental defects in *et al.* (2007). Exposure to Zn and Copper disrupted the neurological system, affecting feeding, swimming and reproductive behaviours in *Daphnia magna* (Pérez & Hoang, 2017). Similarly, the effects of Cd on the growth, reproduction and behaviour of *D. magna* have been thoroughly reported (Vlaeminck *et al.*, 2020). Further, Cd contamination can disrupt aquatic ecosystems, leading to reduced biodiversity and impaired reproductive success in organisms (McGeer *et al.*, 2007). Conversely, Zn is an essential mineral that is involved in various physiological functions, including hormone production, digestion and immunological function (Baltaci *et al.*, 2022). However, it is important to note that Zn can also exert toxic effects when their metal speciation changes, rendering them bioavailable McGeer *D. magna* (Vlaeminck *et al.*, 2020).

The majority of the published studies have primarily addressed the individual toxicity of heavy metals (Tchounwou *et al.*, 2012), with limited research focusing on their combined toxicity. In addition, previous literature primarily focuses on assessing metal mixture interaction types based on mathematical models (Wu *et al.*, 2016). According to Norwood *et al.* (2003), who investigated the mixture effects of metals in aquatic organisms, approximately 70% of these combinations caused additive or antagonistic interactions (Norwood *et al.*, 2003). To date, no comprehensive study has explicitly addressed the combined toxicity of Cd and Zn using freshwater species like *D. magna*.

Water fleas, scientifically known as *et al.* (2020) highlighted *D. magna*, belong to crustaceans inhabiting freshwater environments. Their high sensitivity to environmental stressors and swift reproductive capabilities make them a frequent subject of ecotoxicological investigations (Tyagi *et al.*, 2013). Recognizing *D. magna* as a keystone species in freshwater food webs underscores the importance of comprehending the potential cascading effects of contaminant exposure on this organism (Hamid *et al.*, 2021). Previously, Vlaeminck *D. magna* as an ideal model organism for determining the toxicological effects of the metals on growth and mortality. Additionally, co-exposure of Dissolved Organic Matter (DOM) and Zn also elevates the reproductive toxicity in *D. magna* (Heijerick *et al.*, 2003).

Mixture toxicity evaluation is a challenging and realistic approach to determining the health risks of chemicals. Various single and combined chemical interactions of metals with endogenous antioxidants offer a comprehensive insight into mechanistic toxicity pathways (Hamid *et al.*, 2020). Moreover, this study aims to identify the single and combined toxicity caused by Cd and Zn environment-relevant exposure. To identify and compare developmental toxicity morphological abnormalities and highlight the induction apoptosis using Acridine Orange (AO) staining.

Materials and Methods

Chemicals Preparation and Experimental Design

All the chemical reagents were acquired from AccuStandard, ensuring 99.6% purity grade (New Haven, CT, USA): Cadmium chloride ($CdCl_2$) CAS No. 10108-64-2, Zinc sulfate ($ZnSO_4$)- CAS No. 7733-02-0. $CdCl_2$ and $ZnSO_4$ were dissolved in ultrapure water (ddH_2O) to prepare a stock solution with a concentration of 1 mg/L. The stock solution was subsequently diluted with ultrapure water (18.2 MΩ) to achieve the desired concentrations of a low dose of 10 μg/L and a high dose of 30 μg/L for single exposure experiments for both chemicals. Similarly, for the combined chemical exposure, three respective treatments were conducted comprising of the control group, low (10 μg/L) and high (30 μg/L) dosage groups of both $CdCl_2$ and $ZnSO_4$ mixtures. Total N = 40 *Daphina* were used for each treatment with the codes for control, low dose and high dose (C1-3, LD1-3, HD1-3), respectively.

D. magna Husbandry, Acute Exposure and Sample Collection

D. magna was cultured using a flow-through system at a temperature of 28±0.5°C under a light-dark cycle of 14:10 h light-dark cycle in the hatchery department of the University Malaysia Terengganu (UMT). The daphnids were fed green algae twice daily. Subsequently, healthy adult *Daphnia* were individually put in beakers, containing a 300 mL solution of both single and combined Cd and Zn exposed at specific concentrations. This exposure lasted for a duration of 48 h. All the toxicity experiments were completed based on the OECD Test No. 211 guidelines (OECD, 2012).

All the exposed solutions were changed every day with the new stock solution to maintain the desired mixture concentration. Each treatment was conducted with three independent biological replicates. Following the 48 h exposure period, all the specimens from both the control and treated groups were preserved at -20°C for further analysis.

Primary Developmental Toxicity

During the exposure treatment, developmental toxicity parameters, including heartbeat, mortality, deformities and body weight, were recorded at 12-h intervals. Both the deformities and the heartbeat were recorded via an inverted microscope. Given the rapid heartbeat of *D. magna,* a stopwatch was employed to record the beats per minute accurately. Morphological deformities, mainly including body structure alteration, reduced antenna length, tail bent and carapace disruption, were also meticulously documented.

Acridine Orange (AO) Staining

AO staining is a nucleic acid-based dye staining that is used to detect Apoptosis in tissues (Kari *et al.*, 2022). Both single and joint exposure to Cd and Zn staining methods were followed, as previously explained by Hamid *et al.* (2020). Using a stock solution of 100 g/mL, *D. magna* was dyed after being placed in the solution for 40 min at 28°C. Subsequently, the specimens were thoroughly washed four times with 1× PBS (pH 7.4). The stained samples were dried and transferred onto glass slides for observation of Apoptosis using a 10× inverted fluorescence microscope with Confocal Laser Scanning Microscopy (CLSM).

Statistical Analysis

The developmental toxicity data were analyzed using the GraphPad Prism 10 version. Two-way analysis of variance (ANOVA) was also used to determine the variability among treated groups with a $p<0.05$, which showed statistical significance. Spearman correlation analysis was computed via heat mapper software. Link: (http://www.heatmapper.ca). Lastly, Image J software was also used to determine the fluorescence intensity for apoptosis analysis.

Results

Primary Developmental Toxicity Indices

Mortality Rates

In the present study, 48 h of single exposure to either Cd and Zn at ERCs caused elevated developmental toxicity. Moreover, a dose-dependent increasing trend in developmental toxicity was observed for all treatment groups (Figs. 1a-c). As for single Cd exposure, the low-dose treatment group (10 μg/L) caused mortality at the highest rate of 54.4% at 48 h exposure, followed by 37.8% at 36 h, 22.2% at 24 h and 15.6% at 12 h exposure (Figure 1a). The high-dose treatment group (30 μg/L) also recorded an increase in the mortality rates at 25.6, 32.2, 38.9 and 53.3% at 12, 24, 36 and 48 h. In addition, single Zn exposure caused relatively lower mortality rates of 6.7, 16.7, 40.0 and 47.8%, respectively at 12, 24, 36 and 48 h, in the low-dose treatment group (10 μg/L), while for the high-dose treatment group (30 μg/L), the highest mortality rate was found at 48 h (48.9%), 36 h (43.3%), 24 h (25.6%) and 12 h (7.8%), respectively (Figure 1b).

Similarly, the joint exposure to Cd and Zn caused an increase in the mortality of *D. magna* throughout the acute exposure duration of 12-48 h. In the low exposure treatment group Cd + Zn (10 μg/L), the peak mortality rate observed was at 48 h (52.2%), followed by 36 h (45.6%), 24 h (24.4%) and 12 h (10.0%) (Fig. 1c). Meanwhile, for the high exposure treatment group Cd + Zn (30 μg/L), it was found that the mortality rate increased to 44.4, 51.1, 55.6 and 62.2% respectively at 12, 24, 36 and 48 h, compared to control Fig. (1c).

Heartbeat Rates

The varying heartbeat rates were observed in *D. magna* throughout the acute exposure of 48 h. Although the general trend of heartbeat rates for all treatment groups was constant, failing to induce any significant dose-dependent change (Fig. 1d-f). For example, in the low exposure group of Cd single exposure, the heartbeat rate ranges between 340-440 beats/min, starting from 440, 340, 420 and 340 beats/min, respectively, at 12, 24, 36 and 48 h (Fig. 1d). There was a slight increase in the range between 380-520 beats/min for the high-dose treatment group of Cd single exposure. The increased heartbeat rate was observed at 48 h (520 beats/min), followed by 36 h (440 beats/min), 24 h (380 beats/min) and 12 h (500 beats/min), respectively (Fig. 1d). Similarly, for Zn single exposure, both treatment groups share the heartbeat rate ranges between 340-420 beats/min, at 360, 380, 420 and 360 beats/min, respectively at 12, 24 36 and 48 h exposure in the low-dose treatment group and 420, 340, 360, 380 beats/min in the high-dose treatment group respectively at 12, 24, 36 and 48 h exposure (Fig. 1e).

Similarly, the combined exposure to Cd + Zn showed the heartbeat rate ranges between 360-460 beats/min (Figure 1f). The low-dose treatment group recorded the heartbeat rate at 12 h (400 beats/min), followed by 24 h (360 beats/min), 36 h (360 beats/min) and 48 h (460 beats/min) respectively. Importantly, for the high-dose co-exposure, the heartbeat rates were relatively higher compared to all other treatment groups, i.e., 460, 440, 400 and 460 beats/min, respectively at 12, 24, 36 and 48 h (Figure 1f).

Growth Rates

The growth rate of *D. magna* was measured based on changes in body weight. All exposure groups displayed a similar trend with a dose-dependent increase in growth rate halfway (12-24 h), followed by a decrease over time (24-48 h) (Figs. 1g-i). For single Cd exposure, the low-dose treatment group (10 μg/L) observed the body weight increase from 0.003 g at 12 h-0.004 g at 24 h. After the 24 h interval, the body weight started to decrease to 0.011 g at 48 h exposure and 0.010 g at 36 h exposure. Figure (1g). The high-dose treatment group (30 μg/L) also recorded an increase in the body weight from 0.002, 0.003, 0.009 g, then 0.007, respectively, at 12, 24, 36 and 48 h, compared to control. Furthermore, for Zn exposure, the low-dose treatment group (10 μg/L) found the body weight fluctuated from 0.002 g and 0.003 g at 12 and 24 h-0.003 g and 0.002 at 36 and 48 h, respectively. The high-dose treatment group (30 μg/L) also had fluctuations in body weight observed at 12 h (0.003 g), followed by 24 h (0.009 g), 36 h (0.005) and 48 h (0.001 g), compared to control (Fig. 1h).

Compared to the single exposures, the body weight in the joint exposure of Cd + Zn for the low-dose treatment group showed variations at 12 h (0.0074 g), followed by 24 h (0.0154 g) to 36 h (0.0068 g) and 48 h (0.0019 g) respectively. Figure (1i). On the contrary, for the high-dose mixture treatment group, a decrease in body weight was recorded from 0.0068, 0.0053, 0.0052 and 0.0016 g, respectively, at 12, 24, 36 and 48 h.

Deformity Rates

The single exposures to Cd and Zn induced developmental toxicity that led to morphological changes in *D. magna*. The overall trend displayed a dose-dependent increase in all treatment groups (Figs. 1j-l). As for single Cd exposure, the deformity rates for the low-dose treatment were 16.7, 24.4, 32.2 and 31.11%, respectively, at 12, 24, 36 and 48 h. For the high-dose Cd group, the deformity rates were observed to be the highest among all treatment groups at 51.1% at 36 h, followed by 42.2% at 48 h, 41.1% at 24 h and 32.2% at 12 h. Figure (1j). Similarly, for single Zn exposure, the low-dose treatment group recorded deformity rates of 10.0, 17.8, 28.9 and 36.7% at 12, 24, 36 and 48 h, respectively. The high-dose treatment showed peak deformity rates at 48 h (42.2%), 36 h (35.6%), 24 h (27.8%) and 12 h (23.3%) Fig. (1k).

The co-exposure to Cd + Zn also induh3ced malformations throughout the exposure treatment. In the low exposure group, it was found that the deformities rate increased starting from 14.4, 22.2, 28.9 and then 22.2%, respectively, at 12, 24, 36 and 48 h. Meanwhile, for the high-exposure group, the peak deformities were observed at 48 h (43.3%), 36 h (38.3%), 24 h (18.9%) and 12 h (12.2%) Figure (1l).

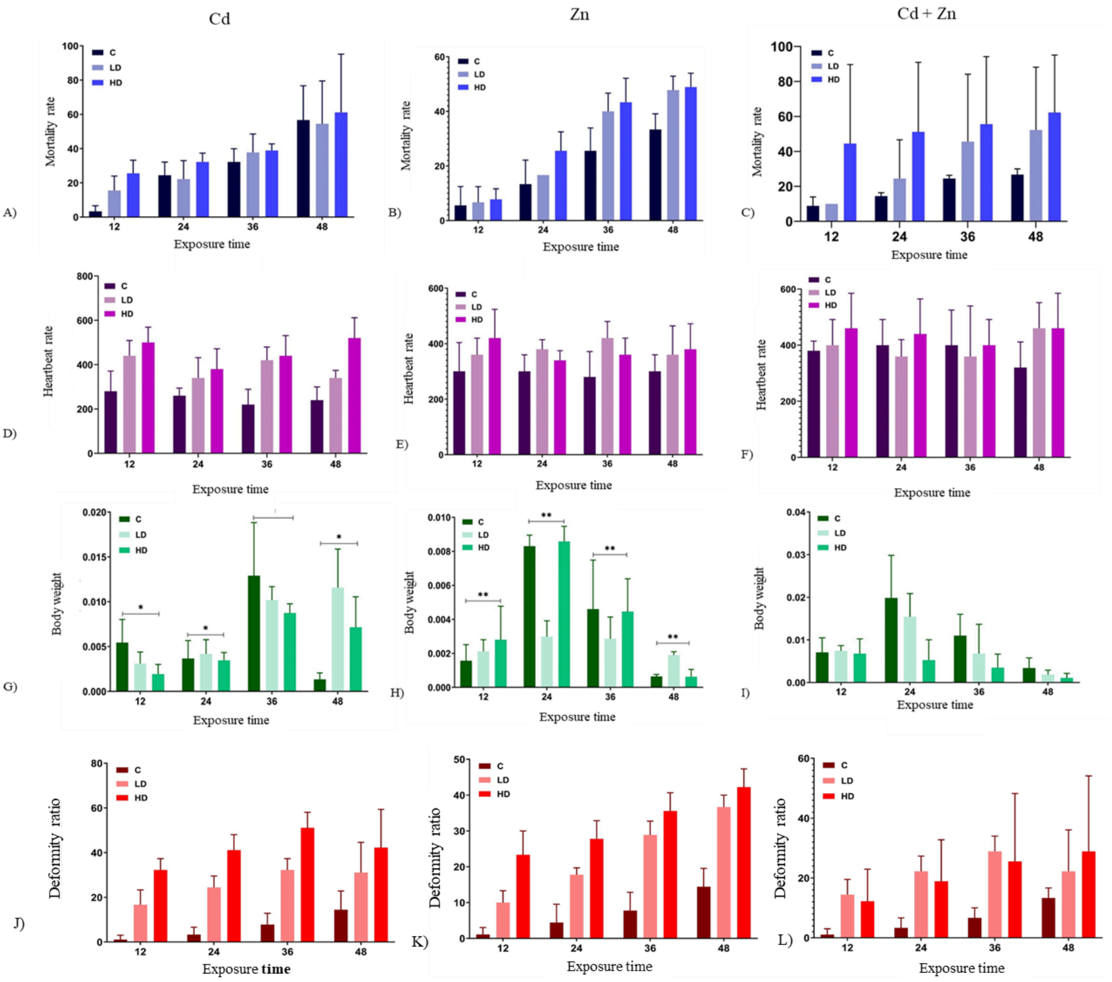

Fig. 1: Graphs representing single and joint Cd + Zn developmental toxicity exposed to D. magna for 48 h post-fertilization (hpf). After acute exposure, developmental lethal (mortality rate and deformity ratio) and non-lethal (heartbeat rate and growth rate) malformations were observed in Environmental Relevant Concentrations (ERCs). The data presented are three replicate groups. Two-way ANOVA significance level: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

DOI: 10.3844/ajessp.2025.39.49

Fig. 2: Malformations of the Daphnia magna after acute exposure of single and joint Cd + Zn at ERCs. Prominent physiological malformations were observed at 12, 24, 36 and 48 h hpf. Abbreviations: SO-Shrunken Organ, SB-Swollen Body, BC-Blood Clotting, ME-Malformed Eyes, PFC-Partially Formed Carapace, DT-Deformed Tail, DI-Darkened Intestines, MT-Missing Tail, EN-Elongated Neck, OT-Overgrown Tail, UA-Underdeveloped Antenna and BA-Bio-Accumulation. LD: Low Dose, HD: High Dose, C: Control. Scale: 400× magnification

Teratogenic Effects

Acute exposure to single and combined Cd and Zn caused serious morphological changes in all treatments (Figure 2). Numerous types of morphological deformities, such as a missing tail, bioaccumulation, blood clotting and an overgrown tail were observed in various treatments, as shown for single Cd exposure (Figure 2a), single Zn exposure (Figure 2b) and joint Cd + Zn exposure (Figure 2c). After single Cd exposure, the majority of the *Daphnia* showed shrinking organs and malformed eyes as early as the 12 h exposure interval.

In addition, some of the Daphnia also showed missing tails and malformed eyes throughout the exposure treatment (Figs. 2b, d-f, m and i). In panel F, it was seen that the Daphnia had a swollen body. Moreover, signs of blood clotting were also found in a dose-dependent manner (Figure 2c, j and l). Similarly, bioaccumulation was observed after 24 h of exposure and it increased over time with the increasing dose (Figure 2e, h and k). At the end of the 48 h, other deformities include an underdeveloped antenna and an overgrown tail (Figure 2l-m).

Figure (2b) displays the morphological defects observed in *Daphnia* after exposure to single Zn exposure. At 12 and 24 h, the major effects were a deformed tail and dose-dependent increase of blood clotting in the low-dose treatment group (Fig. 2b and d) and a partially formed carapace and elongated neck in the high-dose treatment group (Fig. 2c and e). Subsequently, at the 36-48 h exposure intervals, signs of bioaccumulation and overgrown tail started to occur in a dose-dependent manner, as shown in (Fig. 2f, i and h), respectively. Additionally, a swollen body, shrunken organs and malformed eyes were observed (Fig. 2g).

Figure (2c) exhibits the variety of morphological deformities observed after combined Cd and Zn exposure. The first signs of deformities recorded at the 12 h interval were darkened intestines, missing tail and bioaccumulation (Figure 2b-c). Interestingly, bioaccumulation was increased in a dose-dependent manner, although it started at a different time interval and was visible in black colour. For instance, the bioaccumulation in the low-dose treatment group appears starting at 24 h exposure interval (Figure 2d, f, h), while for the high-dose treatment group, the bioaccumulation first observed was at 12 h exposure interval (Figure 2c, e, g, i). By the end of the exposure treatment at 48 h intervals, bioaccumulation can still be observed in the low-dose treatment group along with missing tail deformity (Figure 2h). Meanwhile, the high-dose treatment group found that the deformities at 48 h intervals were malformed eyes and shrunken organs (Figure 2i).

Induction of Apoptosis

Apoptosis detection was performed following the AO staining of the alive deformed *D. magna* (Fig. 3). The anatomy of *D. magna* was observed as the Apoptosis can occur anywhere from its head and antennae, clustered around the abdominal area or throughout the specimen's digestive tract (Fig. 3b). Apoptosis was then measured based on the fluorescence value calculated through the intensity that appears on the stained specimen (Fig. 3c).

For the single Cd exposure, apoptosis cells were found near the head area, as seen in Figure (3b), with a fluorescence value of 46.3 for low-dose and 54.9 for high-dose treatment groups in which the Apoptosis occurred along the length of its digestive tract, respectively. However, with single Zn exposure, apoptosis-mediated fluorescence was observed in the high-dose treatment group and no apoptosis was found in the low-dose treatment group. The Apoptosis was observed at the base of the antennae as well as at the end of its intestines (Figure 3f) and the fluorescence value was recorded at 36.6. On the other hand, for the binary chemical exposure of Cd and Zn, the Apoptosis presents itself clustered around the abdominal area of the specimen (Figure 3g) and also along the length of its antennae. The fluorescence values in the co-exposure groups were observed at 36.8 and 43.6, respectively, for the low-dose treatment and high-dose treatment groups, respectively.

Spearman Correlation

Spearman correlation analysis was used to determine the relationship between the primary developmental toxicity indices and treated groups (Figure 4). The significance of the variables can only be acknowledged if the coefficient of determination, R-value, is at 0.5 and -0.5 or (Table 2). As for Cd single exposure, heartbeat rates vs. deformities rates were significantly positively correlated (R = 0.75), while the rest of the parameters variables showed insignificant correlation (Figure 4a).

In the case of Zn single exposure, the mortality rates vs. deformities rates (R = 0.804) and heartbeat rate vs. deformities rate (R = 0.63) showed highly significant positive correlations. As for the joint exposure to Cd and Zn (Figure 4c), it displayed a range of significantly positive and negative correlations. The mortality rates showed a significant positive relation with the heartbeat rates (R = 0.54) and deformity rates (R = 0.804). In contrast, mortality rates vs. growth rates were found to be significantly negatively correlated at R= -0.61.

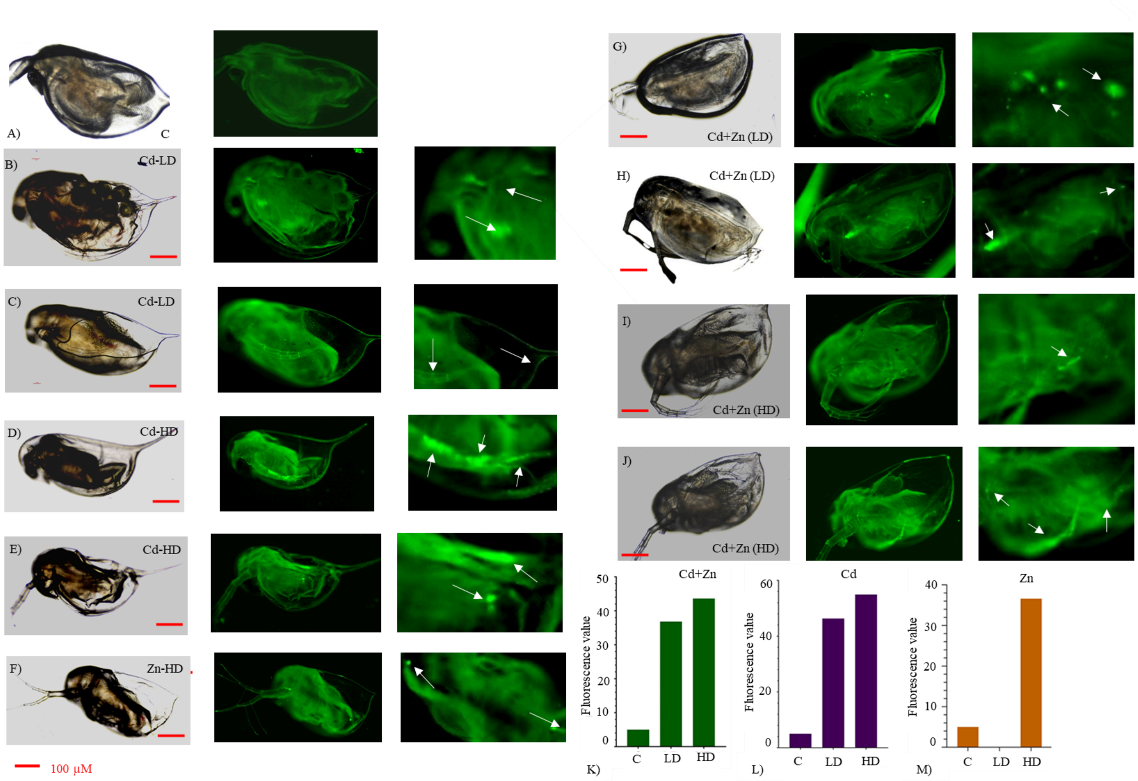

Fig. 3: Acridine orange staining displaying Apoptosis with green fluorescence after acute single and joint exposure of Cd + Zn. All the samples were captured on the bright field and dark field with a scale of 100 µm. (F) Fluorescence intensity values of the Apoptosis were calculated through ImageJ

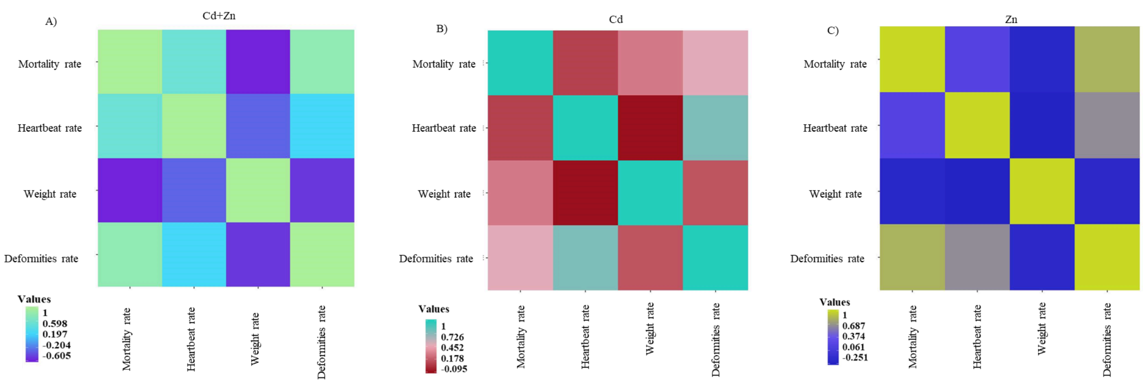

Fig. 4: Spearman correlation via heat maps showing single and joint Cd + Zn developmental toxicity parameters (mortality, body weight, deformities ratio and heartbeat rate) observed after 48 h treatment. Significance levels: $^{*}p<0.05$

Table 1: Comparative literature showing the single and joint Cd + Zn toxicity bioassays in vivo

Chemical	Exposure concentration	Exposure duration	Model species	Toxicity endpoints	References
Cd	0, 0.5, 1, 2 and 5 μg/L	40 days	*Daphnia magna* (water flea)	Cd showed higher mortality with reduced body length and caused reproductive toxicity	Kluttgen and Ratte (1994)
Cd	3.4 μg/L, 31 μg/L,400 μg/L	36, 48, 60, 72 and 96 h	Mice	Male mice were found to be sensitive to low concentrations of cadmium by increased mortality.	Al-Attar (2011)
Zn	340 g/L	21 days	*Daphnia magna* (water flea)	After 1 week, only 7% of the organisms exposed to 340 g/L survived	Muyssen *et al*. (2006)
Zn	13.0-821 μg/L	7 days	*Ceriodaphnia dubia* and *Daphnia carinata*	Increased mortality reproductive toxicity at low exposure levels	Cooper *et al*. (2009)
Zn	0.5, 1, 1.5, 2-6 or 10 mg/L	21 days	*Zebrafish embryos*	Total fecundity and life span were significantly reduced	Lee *et al*. (2021)
Zn	64, 128, 256, 512 and 1024 μg/L for Zn	21 days	*Daphnia magna* (water flea)	Survival and reproduction were significantly reduced	Vlaeminck *et al*. (2020)
Cd + Zn	Cd: 3.4 μg/L-31,400 μg/L Zn: 10.6 and 450.9 μg/L	36, 48, 60, 72 and 96 h	*Daphnia magna* (water flea)	Cadmium is more toxic than zinc, but zinc-cadmium mixtures are less toxic than expected	Attar and Maly (1982)
Cd + Zn	Cd: 0.5, 1.2, 4, 10 μg/L Zn: 10, 100, 200, 400, 1000 μg/L	24 h	*Daphnia magna* (water flea)	Exposure to low concentrations of Zn increased the sublethal tolerance to Cd	Barata *et al*. (2002)
Cd + Zn	Cd: 0.2, 1, 5, 10, 20, 50 and 100 μg/L Zn: 10, 20, 50, 100, 200, 500 and 1,000 μg/L	3 days	*Daphnia magna* (water flea)	Low concentrations of Cd or Zn exposure increased the SOD activity	Fan *et al*. (2009)

Table 2: Spearman correlation results displaying the relationship between developmental toxicity parameters after single and joint Cd + Zn exposure in Daphnia Magna

Parameters	Mortality rate	Heartbeat rate	Body weight	Deformities rate
Single Cd exposure				
Mortality rate	1	0.10	0.29	0.48
Heartbeat rate	0.10	1	-0.09	0.75
Body weight	0.29	-0.09	1	0.15
Deformity rate	0.48	0.75	0.15	1
Single Zn exposure				
Mortality rate	1	0.19	-0.19	0.80
Heartbeat rate	0.19	1	-0.25	0.63
Body weight	-0.19	-0.25	1	-0.16
Deformity rate	0.80	0.63	-0.16	1
Combined Cd + Zn exposure				
Mortality rate	1	0.54	-0.60	0.80
Heartbeat rate	0.54	1	-0.29	0.23
Body weight	-0.60	-0.29	1	-0.49
Deformity rate	0.80	0.23	-0.49	1

Discussion

In environmental settings, various chemicals coexist due to varying sources and contamination histories (Xu *et al.*, 2024). Therefore, the mixture toxicity assessment of chemicals is an important realistic method for evaluating the toxicological effects of pollutants on aquatic species (Hamid *et al.*, 2021). Previously, various studies have been highlighted the single metal toxicological effects in aquatic species. (Table 1). The present study highlights the single and combined developmental toxic effects of Cd and Zn in *et al.* (2017) also recorded up to a 70% decrease in *et al.* (2006), which also reported a significant increase in *D. magna* after acute exposure to environmentally relevant levels (10 and 30 μg/L). The majority of the treated groups showed a dose-dependent increase in the mortality rate for both single and combined exposures. Martins *D. magna* survivability at a Zn concentration (4000 μg/L) much higher than that we employed in the current study. Previous studies have also shown higher mortality with reduced body length in *D. magna* after Cd exposure (Kluttgen & Ratte, 1994; Muyssen *et al.*, 2006). Similarly, the high-dose group exhibited a higher mortality rate compared to the low-dose group at each time interval. This finding is consistent with the results of a study by Shaw *D. magna* mortality rates with increasing Cd concentrations. However, previous studies highlighted the mortality rates of metals after a single exposure and we highlight both the single and combined impacts of metals on mortality rates in the current investigation.

Observing the heartbeat rate is crucial as it serves as one of the initial indicators of the body's response to chemical exposure. In this study, the results demonstrated a consistent increase in heartbeat rates of *D. magna*, maintained within a specific range of beats per minute throughout the exposure period. This finding contrasts with previous research studies; a decrease in heart rate and thoracic limb activity was reduced by 4.3-11.7 and 5.0-10.3%, respectively, in all Cd treatments compared to the control (Wei *et al.*, 2022). In addition, a concentration-dependent decrease in heartbeat rate was also found after ternary exposure to metals including Copper, Nickel and Zn in *D. magna* (Park *et al.*, 2019).

In this study, the growth rate was observed by monitoring *et al.* (2022), who observed a similar pattern in the body weight of *Daphnia*'s body weight, which showed a dose-dependent increase until 24 h of exposure, followed by a decreasing trend halfway through the 48-h exposure duration in all treatments. This observation can be supported by a study by Wei *D. magna* as the dosage concentrations increased, followed by a significant decrease after the 21 days of exposure compared to the control treatment. Another study examining single Zn exposure intake observed a direct correlation between the concentration of the chemical and the body weight of *D. magna*. This finding suggests the increased exposure concentration elevates the bioaccumulation of Zn within the organisms (Heugens *et al.*, 2006).

In contrast, Fan *et al.* (2009) observed a dose-dependent increase in the combined Cd-Zn mixture exposure, suggesting a synergistic effect (Fan *et al.*, 2009), whereas Berglind (1986) highlighted the binary mixtures of metal that had no significant effects on *Daphnia* growth after 8 days of exposure compared to the control treatments (Berglind, 1986). Comparing the single and combined treatment groups, it was evident that the growth rate of *D. magna* in the Cd-Zn mixture treatments surpassed that of the single treatment groups.

In this study, both single and combined treatment groups of Cd and Zn showed similar types of morphological deformities with a time and dose-dependent increase in their severity. The deformity types consist of shrunken organs, swollen bodies, blood clotting, malformed eyes, underdeveloped antennae, missing tails, overgrown tails, deformed tails, partially formed carapaces, elongated necks and darkened intestines. Similarly, a dose-dependent increase in the deformity rate of the zebrafish development when exposed to Zn chemicals in different concentrations (Yang *et al.*, 2021). Furthermore, Xu *et al.* (2024) investigated the effects of Cd mixture exposure on the swim bladder histology of Japanese medaka (Oryzias latipes) and observed that the highest occurrence of deformities in the uninflated swim bladder was associated with Cd exposure. Another study observed malformations such as tail curving, yolk sac oedema and pericardial oedema after zebrafish embryos and larvae were exposed to Zn and Nickel mixture (Yang *et al.*, 2021). This indicates that the presence of contaminants in freshwater environments inhibits the development of the species.

Apoptosis serves as a sensitive indicator of cellular stress, often affected by chemical exposure (Hamid *et al.*, 2022). In our study, both the single Cd and Zn and the joint Cd + Zn exposure groups exhibited elevated levels of Apoptosis, highlighting the impact of these treatments on cellular health. The majority of the Apoptosis was observed around the head, eyes and antennae area, as well as along its digestive tract. Similar findings were observed regarding epithelial cells in the midgut region when polystyrene microplastics were exposed to Artemia (Suman *et al.*, 2020). In short, single and joint Cd and Zn have the potential to disrupt the equilibrium of the antioxidant system, leading to oxidative damage and increased Apoptosis.

Spearman correlation results found a significant positive correlation between heartbeat and deformity rates after Cd exposure. Meanwhile, the treated group exhibited a positive correlation between the deformities rate and mortality rate variables. Spearman's correlation has also been utilized in previous studies to assess the significant relationship between the body growth, mortality, deformity and body length variables after exposure to the phthalate's binary mixtures in zebrafish (Hamid *et al.*, 2020), where it was found that the majority of the variables were strongly correlated and cause reproductive toxicity. Meanwhile, a significantly negative correlation ($R^2 = -0.61$) was observed between the mortality rate and growth rate for the combined Cd-Zn toxicity exposure. This negative correlation suggests that as the mortality rate increases, the growth rate decreases, indicating that Zn exerts a sublethal effect during acute Cd exposure. In summary, the combined exposure of Cd and Zn exhibited higher toxicity potential than individual exposures to Cd and Zn, indicating synergistic toxic effects in *D. magna*.

Conclusion

In conclusion, Cd and Zn caused developmental toxicity to *Daphnia magna* based on their mortality, heartbeat, growth and developmental deformities after environmental level exposure. In comparison with the single exposure groups, the binary metal mixture treatment group was found most toxic with the highest rate of mortality in a dose-dependent manner. Moreover, various morphological defects such as missing tails, antennae, shrinking organs and bioaccumulation were dominant in all treated groups. Apoptosis was evident through AO staining, revealing bright green fluorescence in the head, antennae and gut areas, indicating the induction of cell death. These findings highlight the severe developmental impacts and Apoptosis induced by single and combined exposures to Cd and Zn at environmental levels.

Acknowledgment

The authors are sincerely thankful for the Talent and Publication Enhancement Research Grant for funding this study.

Funding Information

This study was funded by the Talent and Publication Enhancement Research Grant, University Malaysia Terengganu (TAPERG/2023/UMT/2422) to NH, Chongqing Technology Innovation and Application Development Sichuan-Chongqing Special Key Project (CSTB2024TIAD-CYKJCXX0017) and High-level Talents Project of Chongqing Medical University (No. R4014).

Author's Contributions

Naima Hamid: Funding sources and manuscript writing.

Siti Nur Airina Binti Ibrahim: Experimentations.

Muhammad Junaid: Statistical analysis, Revision.

De-Sheng Pei: Conceptualization and supervision.

Ethics

All the experiments associated with *D. magna* were performed with compliance from the Ethics Committee of the University Malaysia Terengganu (UMT), Malaysia. The Ethical approval ID is UMT/JKEPHMK/2023/107.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data will be provided upon reasonable request.

References

Al-Attar, A. M. (2011). Antioxidant Effect of Vitamin E Treatment on Some Heavy Metals-Induced Renal and Testicular Injuries in Male Mice. *Saudi Journal of Biological Sciences*, *18*(1), 63-72. https://doi.org/10.1016/j.sjbs.2010.10.004

Attar, E. N., & Maly, E. J. (1982). Acute Toxicity of Cadmium, Zinc, and Cadmium-Zinc Mixtures Todaphnia Magna. *Archives of Environmental Contamination and Toxicology*, *11*(3), 291-296. https://doi.org/10.1007/bf01055205

Baltaci, S. B., Unal, O., Gulbahce-Mutlu, E., Gumus, H., Pehlivanoglu, S., Yardimci, A., Mogulkoc, R., & Baltaci, A. K. (2022). The Role of Zinc Status on Spatial Memory, Hippocampal Synaptic Plasticity, and Insulin Signaling in icv-STZ-Induced Sporadic Alzheimer's-Like Disease in Rats. *Biological Trace Element Research*, *200*(9), 4068-4078. https://doi.org/10.1007/s12011-021-02999-2

Barata, C., Markich, S. J., Baird, D. J., Taylor, G., & Soares, A. M. V. M. (2002). Genetic Variability in Sublethal Tolerance to Mixtures of Cadmium and Zinc in Clones of Daphnia Magna Straus. *Aquatic Toxicology*, *60*(1-2), 85-99. https://doi.org/10.1016/s0166-445x(01)00275-2

Berglind, R. (1986). Combined and Separate Effects of Cadmium, Lead and Zinc on ALA-D Activity, Growth and Hemoglobin Content in*Daphnia MagnaEnvironmental Toxicology and Chemistry*, *5*(11), 989-995. https://doi.org/10.1002/etc.5620051107

Cooper, N. L., Bidwell, J. R., & Kumar, A. (2009). Toxicity of Copper, Lead, and Zinc Mixtures to Ceriodaphnia Dubia and Daphnia Carinata. *Ecotoxicology and Environmental Safety*, *72*(5), 1523-1528. https://doi.org/10.1016/j.ecoenv.2009.03.002

Cukrov, D. Sc. N., Cuculić, D. Sc. V., & Kwokal, D. Sc. Ž. (2008). Ecotoxic Metals in Water and Sediment of the Southeastern Part of the of Šibenik Harbor, Croatia. *Third International Conference on Ports and Waterways-POWA*, 278-286.

Fan, W.-H., Tang, G., Zhao, C.-M., Duan, Y., & Zhang, R. (2009). Metal Accumulation and Biomarker Responses in *Daphnia Magna* Following Cadmium and Zinc Exposure. *Environmental Toxicology and Chemistry*, *28*(2), 305-310. https://doi.org/10.1897/07-639.1

Garcia-Santos, S., Monteiro, S., Malakpour-Kolbadinezhad, S., Fontaínhas-Fernandes, A., & Wilson, J. (2015). Effects of Cd Injection on Osmoregulation and Stress Indicators in Freshwater Nile Tilapia. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *167*, 81-89. https://doi.org/10.1016/j.cbpc.2014.09.002

Gong, B., He, E., Van Gestel, C. A. M., Tang, Y., Yang, W., Yang, J., Li, Y., & Qiu, H. (2021). Dynamic Interaction Processes of Rare Earth Metal Mixtures in Terrestrial Organisms Interpreted by Toxicokinetic and Toxicodynamic Model. *Journal of Hazardous Materials*, *418*, 126281. https://doi.org/10.1016/j.jhazmat.2021.126281

Hamid, N., Junaid, M., Manzoor, R., Duan, J.-J., Lv, M., Xu, N., & Pei, D.-S. (2022). Tissue Distribution and Endocrine Disruption Effects of Chronic Exposure to Pharmaceuticals and Personal Care Products Mixture at Environmentally Relevant Concentrations in Zebrafish. *Aquatic Toxicology*, *242*, 106040. https://doi.org/10.1016/j.aquatox.2021.106040

Hamid, N., Junaid, M., Manzoor, R., Jia, P.-P., & Pei, D.-S. (2020). Prioritizing Phthalate Esters (PAEs) Using Experimental in Vitro/Vivo Toxicity Assays and Computational in Silico Approaches. *Journal of Hazardous Materials*, *398*, 122851. https://doi.org/10.1016/j.jhazmat.2020.122851

Hamid, N., Junaid, M., & Pei, D.-S. (2021). Combined Toxicity of Endocrine-Disrupting Chemicals: A Review. *Ecotoxicology and Environmental Safety*, *215*, 112136. https://doi.org/10.1016/j.ecoenv.2021.112136

Harding, J. P. C., & Whitton, B. A. (1978). Zinc, Cadmium and Lead in Water, Sediments and Submerged Plants of the Derwent Reservoir, Northern England. *Water Research*, *12*(5), 307-316. https://doi.org/10.1016/0043-1354(78)90118-5

Heijerick, D. G., Janssen, C. R., & Coen, W. D. (2003). The Combined Effects of Hardness, pH, and Dissolved Organic Carbon on the Chronic Toxicity of Zn to D. magna: Development of a Surface Response Model. *Archives of Environmental Contamination and Toxicology*, *44*(2), 0210-0217. https://doi.org/10.1007/s00244-002-2010-9

Heugens, E. H. W., Tokkie, L. T. B., Kraak, M. H. S., Hendriks, A. J., van Straalen, N. M., & Admiraal, W. (2006). Population Growth of*Daphnia magna*Under Multiple Stress Conditions: Joint Effects of Temperature, Food, and Cadmium. *Environmental Toxicology and Chemistry*, *25*(5), 1399-1407. https://doi.org/10.1897/05-294r.1

Kari, S., Subramanian, K., Altomonte, I. A., Murugesan, A., Yli-Harja, O., & Kandhavelu, M. (2022). Programmed Cell Death Detection Methods: A Systematic Review and a Categorical Comparison. *Apoptosis*, *27*(7), 482-508. https://doi.org/10.1007/s10495-022-01735-y

Kluttgen, B., & Ratte, H. T. (1994). Effects of Different Food Doses on Cadmium Toxicity to *Daphnia MagnaEnvironmental Toxicology and Chemistry*, *13*(10), 1619-1627. https://doi.org/10.1002/etc.5620131011

Lee, G., Lee, B., & Kim, K.-T. (2021). Mechanisms and Effects of Zinc Oxide Nanoparticle Transformations on Toxicity to Zebrafish Embryos. *Environmental Science: Nano*, *8*(6), 1690-1700. https://doi.org/10.1039/d1en00305d

Martins, C., Jesus, F. T., & Nogueira, A. J. A. (2017). The Effects of Copper and Zinc on Survival, Growth and Reproduction of the Cladoceran Daphnia Longispina: Introducing New Data in an "Old" Issue. *Ecotoxicology*, *26*(9), 1157-1169. https://doi.org/10.1007/s10646-017-1841-0

McGeer, J. C., Nadella, S., Alsop, D. H., Hollis, L., Taylor, L. N., McDonald, D. G., & Wood, C. M. (2007). Influence of Acclimation and Cross-Acclimation of Metals on Acute Cd Toxicity and Cd Uptake and Distribution in Rainbow Trout (Oncorhynchus Mykiss). *Aquatic Toxicology*, *84*(2), 190-197. https://doi.org/10.1016/j.aquatox.2007.03.023

Muyssen, B. T. A., De Schamphelaere, K. A. C., & Janssen, C. R. (2006). Mechanisms of Chronic Waterborne Zn Toxicity in Daphnia Magna. *Aquatic Toxicology*, *77*(4), 393-401. https://doi.org/10.1016/j.aquatox.2006.01.006

Norwood, W. P., Borgmann, U., Dixon, D. G., & Wallace, A. (2003). Effects of Metal Mixtures on Aquatic Biota: A Review of Observations and Methods. *Human and Ecological Risk Assessment: An International Journal*, *9*(4), 795-811. https://doi.org/10.1080/713610010

OECD. (2012). *Daphnia magna Reproduction Test*.

Pan, J., Plant, J. A., Voulvoulis, N., Oates, C. J., & Ihlenfeld, C. (2010). Cadmium Levels in Europe: Implications for Human Health. *Environmental Geochemistry and Health*, *32*(1), 1-12. https://doi.org/10.1007/s10653-009-9273-2

Park, S., Jo, A., Choi, J., Kim, J., Zoh, K.-D., & Choi, K. (2019). Rapid Screening for Ecotoxicity of Plating and Semiconductor Wastewater Employing the Heartbeat of Daphnia Magna. *Ecotoxicology and Environmental Safety*, *186*, 109721. https://doi.org/10.1016/j.ecoenv.2019.109721

Pérez, E., & Hoang, T. C. (2017). Chronic Toxicity of Binary-Metal Mixtures of Cadmium and Zinc to *Daphnia MagnaEnvironmental Toxicology and Chemistry*, *36*(10), 2739-2749. https://doi.org/10.1002/etc.3830

Shaw, J. R., Dempsey, T. D., Chen, C. Y., Hamilton, J. W., & Folt, C. L. (2006). Comparative toxicity of Cadmium, Zinc, and Mixtures of Cadmium and Zinc to Daphnids. *Environmental Toxicology and Chemistry*, *25*(1), 182-189. https://doi.org/10.1897/05-243r.1

Shephard, B. K., McIntosh, A. W., Atchison, G. J., & Nelson, D. W. (1980). Aspects of the Aquatic Chemistry of Cadmium and Zinc in a Heavy Metal Contaminated Lake. *Water Research*, *14*(8), 1061-1066. https://doi.org/10.1016/0043-1354(80)90153-0

Suman, T. Y., Jia, P.-P., Li, W.-G., Junaid, M., Xin, G.-Y., Wang, Y., & Pei, D.-S. (2020). Acute and Chronic Effects of Polystyrene Microplastics on Brine Shrimp: First Evidence Highlighting the Molecular Mechanism Through Transcriptome analysis. *Journal of Hazardous Materials*, *400*, 123220. https://doi.org/10.1016/j.jhazmat.2020.123220

Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K., & Sutton, D. J. (2012). Heavy Metal Toxicity and the Environment. *Molecular, Clinical and Environmental Toxicology: Volume 3: Environmental Toxicology*, 133-164. https://doi.org/10.1007/978-3-7643-8340-4_6

Tyagi, R., Rana, P., Gupta, M., Khan, A. R., Bhatnagar, D., Bhalla, P. J. S., Chaturvedi, S., Tripathi, R. P., & Khushu, S. (2013). Differential Biochemical Response of Rat kidney Towards Low and High Doses of $NiCl_2$ as Revealed by NMR Spectroscopy. *Journal of Applied Toxicology*, *33*(2), 134-141. https://doi.org/10.1002/jat.1730

Vlaeminck, K., Viaene, K. P. J., Van Sprang, P., & De Schamphelaere, K. A. C. (2020). Development and Validation of a Mixture Toxicity Implementation in the Dynamic Energy Budget-Individual-Based Model: Effects of Copper and Zinc on *Daphnia magna* Populations. *Environmental Toxicology and Chemistry*, *40*(2), 513-527. https://doi.org/10.1002/etc.4946

Wei, X., Li, X., Liu, H., Lei, H., Sun, W., Li, D., Dong, W., Chen, H., & Xie, L. (2022). Altered Life History Traits and Transcripts of Molting- and Reproduction-Related Genes by Cadmium in Daphnia Magna. *Ecotoxicology*, *31*(5), 735-745. https://doi.org/10.1007/s10646-022-02541-7

Wu, X., Cobbina, S. J., Mao, G., Xu, H., Zhang, Z., & Yang, L. (2016). A Review of Toxicity and Mechanisms of Individual and Mixtures of Heavy Metals in the Environment. *Environmental Science and Pollution Research*, *23*(9), 8244-8259. https://doi.org/10.1007/s11356-016-6333-x

Xu, Y., Talukder, M., Li, C., Zhao, Y., Zhang, C., Ge, J., & Li, J. (2024). Nano-Selenium Alleviates Cadmium-Induced Neurotoxicity in Cerebrum Via Inhibiting Gap Junction Protein Connexin 43 Phosphorylation. *Environmental Toxicology*, *39*(3), 1163-1174. https://doi.org/10.1002/tox.24001

Yang, Y., Yu, Y., Zhou, R., Yang, Y., & Bu, Y. (2021). The Effect of Combined Exposure of Zinc and Nickel on the Development of Zebrafish. *Journal of Applied Toxicology*, *41*(11), 1765-1778. https://doi.org/10.1002/jat.4159