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Accepted Article

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Chronic toxicity of metal mixtures to *D. magna*

CHRONIC TOXICITY OF BINARY-METAL MIXTURES OF CADMIUM AND ZINC TO *DAPHNIA MAGNA*

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This article contains online-only Supplemental Data

Abstract

The present study characterized the chronic effect of binary-metal mixtures of cadmium (Cd) and zinc (Zn) on *Daphnia magna*. The titration design was chosen to characterize the 21-d chronic effects of the binary-metal mixtures on survival, growth, reproduction, and metal accumulation in *D. magna*. Using this design, increasing concentrations of Zn (10, 20, 40, 80, 120, 160 and 200μg/L) were titrated against a constant concentration of 1.5μg/L Cd. The results demonstrated that Cd was highly toxic to *D. magna*. In a mixture with Cd and Zn, sublethal concentrations of 10 and 20μg/L Zn were insufficient to protect *D. magna* from chronic Cd toxicity, while mixtures containing 40, 80, and 120μg/L Zn provided strong protective effects to *D. magna* at all endpoints and resulted in less-than-additive effects. At higher Zn concentrations, such as 160 and 200μg/L, Zn appeared to contribute to the toxicity. The less-than-additive effects observed in the Cd-Zn mixture can be explained by the decrease in body Cd concentration when increasing Zn concentration in the exposure media. Embryos analyzed for morphological alterations in the Cd-Zn mixtures demonstrated severe developmental defects. The effect of Cd on undeveloped embryos while both Zn and Cd are present in the organisms raises a question of whether the competitive binding mechanism of Zn and Cd is still happening at the cellular level in the organisms. The results of the present study are useful for the development of the Biotic Ligand Model and environmental quality guidelines for metal mixtures. This article is protected by copyright. All rights reserved

Keywords: metal toxicity; metal mixture toxicity; reproductive toxicity; embryotic development; metal mixture uptake; *Daphnia magna*

INTRODUCTION

In nature, aquatic ecosystems are generally contaminated with mixtures of multiple metals. Studies have investigated metal mixture effects for decades [1] but a great portion of those studies have only invested large efforts in understanding acute toxicity. For example, in recent meta-analyses conducted by Norwood et al. [2] and Vijver et al. [3] only 35% and 9% of the studies respectively, reported chronic toxicity. A recent metal mixture evaluation conducted by Meyer et al. [1] indicated the need for chronic mixture studies since the lack of chronic toxicity data in the literature makes it difficult to arrive at meaningful deductions concerning the interactions of metals in long-term exposures. As a result, studies investigating chronic toxicity of metal mixtures at sublethal concentrations are essential to improve scientific understanding of interactions between metals and organisms and long-term metal mixture toxicity.

Of interest in the present study is the chronic mixture toxicity of cadmium (Cd) and zinc (Zn). Cadmium and Zn are two metals that are commonly found in aquatic environments [4] especially near metal-mining and/or metal-processing facilities. Cadmium is a trace non-essential metal that has no biological function and is very toxic to aquatic life [5, 6]. Cadmium enters the aquatic environment from both natural sources (chemical weathering of soils and bedrock) and anthropogenic sources (mining, leachate from contaminated sites, and industrial wastewater discharges etc.) [7]. Zinc on the other hand, is an essential nutrient for biotic life, but is also toxic at high concentrations [8, 9]. Zinc is introduced into aquatic environments through various anthropogenic (manufacturing of brass and other alloys, automobile equipment, medical tools, domestic appliances, pharmaceuticals, construction, and synthesis of animal feed and fertilizers), and natural sources (chemical weathering of rocks and minerals) [10].

The acute toxicity of metal mixtures on aquatic organisms is not consistent. Studies examining the effect of Cd and Zn mixtures across *Daphnia* species, and freshawater shrimp and copepods have shown additive (noninteractive) and nonadditive (interactive) effects [2, 11-13]. Norwood et al. [2] proposed that the variability in organism responses might just be due to concentration-dependent, and inter/intraspecific species differences in sensitivity. In addition, random variability in organism responses may have led to inaccurate interpretation of mixture toxicity assessments as demonstrated by De Laender et al. [14]. If both metal alone and metal mixture toxicity tests are not simultaneously conducted, truly nonadditive (less-than-additive or greater-than-additive) effects might be misinterpreted as additive effects, or vice versa. As a result, in the present study, it was crucial and essential that both Zn alone and Cd-Zn mixture tests were conducted concurrently to avoid misleading toxicity interpretations.

The objective of the present study is to characterize the chronic effect of Cd and Zn mixtures using a titration experimental design as used by Meyer et al. [13]. The measured endpoints include: survival, reproduction, growth, and metal accumulation in *D. magna* when exposed to Zn alone and to mixtures of Cd-Zn at sublethal concentrations.

MATERIALS AND METHODS

Test organisms

Laboratory cultured *Daphnia magna* from the Ecotoxicology and Risk Assessment Laboratory at the Loyola University Chicago's Institute of Environmental Sustainability (IES) were utilized in this study. *Daphnia magna* cultures were maintained in 1L glass beakers (30 per beaker). Beakers were filled with 900-1000 ml reconstituted moderately hard water (MHW) made from 16-18MΩ MilliQ water and laboratory grade chemicals ($CaSO_4.2H_2O$, $MgSO_4$, $NaHCO_3$, and KCl) based on the U.S.EPA method for chronic toxicity testing [15]. The water

quality measures for the culture media were as follows: hardness ranged from 80 to 84 mg/L as CaCO$_3$, alkalinity ranged from 55 to 61 mg/L as CaCO$_3$, pH ranged from 7.19 to 7.89, and dissolved oxygen (DO) ranged from 6.87 to 8.32 mg/L. Cultures were maintained at a temperature ranging from 21.2 to 25.6°C and at a light:dark photoperiod of 16h:8h. *Daphnia magna* culture water was changed on Mondays, Wednesdays, and Fridays. Dissolved oxygen, pH, and temperature were recorded following subsequent water changes. Dissolved oxygen and temperature were measured with a DO instrument: model YSI 550A, and pH was measured with a Fisher Scientific accumet portable laboratory meter: model AP110. Cultured *D. magna* were fed 6ml of an algae suspension (*Raphidocelis subcapitata,* formerly known as *Selenastrum capicornutum*) at a concentration of 3×10^7 cells/ml, and 3ml YCT (a food suspension of yeast, cereal leaves and trout chow) at a concentration of 1.7- 1.9 mg solids/L daily. The algae and YCT were cultured and prepared in the Loyola University Chicago's Ecotoxicology and Risk Assessment Laboratory based on the U.S. EPA Method [15].

Metal alone and binary-metal toxicity tests

The titration method was used in this study to characterize the chronic toxicity of Cd-Zn mixtures on *D. magna*. The titration design was chosen because it affords the detection of graded changes in organism responses, across graded increases in metal concentrations [13]. Using this design, nominal Cd concentration was kept constant at 1.5µg/L across all treatments and nominal Zn concentrations varied (10, 20, 40, 80, 120, 160 and 200 µg/L). Concentrations of Cd and Zn were chosen based on screening chronic toxicity tests conducted in our laboratory (unpublished data). A control (MHW) was also used. To compare with the mixture effect, a chronic Zn alone test with concentrations equal to the Zn concentrations in the mixture test was conducted. A twenty-one day (21) static renewal toxicity test method was used in the present study [16]. To

avoid potential interference of organism health effect, the Zn alone and mixture tests were conducted simultaneously using neonate *D. magna* coming from the same batch (5th brood). Tests were conducted with 48 h test solution renewals in an aquatic toxicology testing room located in the Loyola IES. The aquatic toxicology testing room was set at a temperature of 21°C and a photoperiod of light:dark = 16h:8h. The exposure media were prepared from stock solutions that were prepared from lab grade metal salts ($CdSO_4$ and $ZnCl_2$). The Zn alone toxicity test consisted of 7 treatments with 4 replicates each while the binary-metal toxicity test consisted of 8 treatments with 4 replicates each which included a Cd alone treatment. Replicates were comprised of 600ml polypropylene cups containing 10 neonate *D. magna* (<24 h old) in 200ml of test solution from day 0 to day 10, and 350ml test solution from day 11 to day 21 to compensate for *D. magna* growth.

Due to the nature of the study, organisms grew over time, therefore, varying amounts of a suspension of *Raphidocelis subcapitata* with a concentration of $3x10^7$ cells/ml and YCT of a concentration of 1.7 – 1.9 mg solids/L were fed daily. Food rations were adjusted depending on the feeding rate of the organisms. Feeding rate was low when organisms were small but increased when the organisms grew bigger (Table 1, Supplemental Data,).

Mortality was recorded daily to determine effects of Zn and Cd-Zn mixture on survival of *D. magna*. A *D. magna* was considered dead if no mobility was observed after gentle probing with a pipette, and if no organ movements were observed using a 6X Bausch & Lomb magnifier (Bausch & Lomb). Dead organisms were removed from the test chambers and discarded. Reproductive output was also observed daily once *D. magna* became sexually mature and began reproducing. All neonates, both dead and alive, were separately counted, recorded, removed from the test chambers, and discarded. The same procedure was used if any undeveloped

embryos were observed in the test chambers. The embryos were collected in a 30 mL polypropylene beaker for morphological examination with a Zeiss ZEN imaging software model SteREO Discovery.v12. Reproductive rate per day was calculated as a ratio of number of neonates produced in a day to the number of surviving adults on the same day. Proportion of dead neonates was calculated as a percentage of number of dead neonates observed in a day over the total number of dead and live neonates counted on the same day. Proportion of undeveloped embryos was calculated as a percentage of the total number of embryos observed in a day over the total number of dead, live neonates, and undeveloped embryos counted on the same day.

Twenty-four hours prior to the termination of the experiment, 50mL polypropylene digestion tubes were labelled, placed in a Fisher Drying Oven, and dried at 60°C. At the end of the experiment, all surviving adults in each test chamber were collected, transferred into a respectively labelled digestion tube, and carefully rinsed three times with DI water. All organisms in each replicate were jointly weighed in the digestion tube with a Metro Toledo Balance Model XS64, and their wet-weight was recorded. Organisms were then dried in the same oven at 60°C for 48 h and reweighed to determine dry weight. The average dry weight for each individual organism was obtained by dividing the total weight in each replicate by the number of surviving organisms in that replicate.

Water quality and chemical analyses

Two 190L batches of MHW were prepared in the Ecotoxicology and Risk Assessment Lab during the Cd-Zn mixtures study as described in the test organism section above. Water quality measures for both toxicity tests (Zn alone and Cd-Zn mixtures) were taken once a week on days 0, 7, 14, and 21. Dissolved oxygen and temperature were measured with a dissolved oxygen instrument: model YSI 550A. The pH was measured with a Fisher Scientific accumet

portable laboratory meter: model AP110. Hardness and alkalinity were measured by titration methods (Methods 2320, 2340) [17]. Hardness was titrated against 0.01 M ethylenediaminetetraacetic acid. Alkalinity was titrated against 0.02 N H_2SO_4. Water samples for total and dissolved metals, cations and anions were also collected weekly and at the same time with collection of water samples for water quality measurements. For each sample type, approximately 14ml of test water was collected into a 15ml polypropylene sample vial. Total metal samples were taken directly from the test water, but dissolved metal and cation and anion samples were filtered through Whatman polyvinyldifluoride filters with a pore diameter of 0.45µm. Water samples for total and dissolved metals were preserved with one drop of concentrated nitric acid, and refrigerated for later analysis. In addition, approximately 25 ml of test water was filtered through a new filter of the same filter type used for dissolved metal and cations and anions and collected into a 30 mL amber glass bottle for analysis of dissolved organic carbon (DOC). To determine body metal content and accumulation, surviving daphnids were digested with HNO_3 based on U.S. EPA Method 3050B [18]. Digested solutions were used for analysis of metal content in surviving daphnids.

Analyses of total metal, dissolved metal, and cation concentrations were conducted using a 300X NexION ICP-MS (Perkin Elmer). Concentrations of DOC were analyzed with a TOC-L CSH analyzer (Shimazu). An 881 Compact Ion Chromatography (Metrohm USA) was used to analyze concentrations of anions. Results of the QC samples showed recovery of 90 to 115% for total and dissolved metals; 95 to 113% for DOC, and 90 to 110% for anions.

Data analyses

For Zn alone test, data were compared for statistically significant differences between control and exposure treatments. For the mixture test in addition to comparisons between control

and exposure treatments, data were also compared for statistically significant differences between Cd alone and Cd-Zn mixture treatments.

A one-way analysis of variance (ANOVA), along with a Tukey's honestly significant difference (HSD) multiple comparisons test was used to detect treatment differences within each endpoint. Differences across treatments of Zn alone and their corresponding Cd-Zn mixtures (e.g., treatment 1 for Zn alone and treatment 2 for Cd-Zn mixture) were also analyzed using a one-way ANOVA. To test for interaction effects of Cd and Zn, a two-way ANOVA was used, treating metal exposure type as a factor (Zn alone or its corresponding Cd-Zn mixture) and the concentrations of zinc as another factor. To meet the requirements of homogenous variance and normal distribution, an arcsine square root transformation method was used for mortality, the proportion of dead neonates and undeveloped embryos data. Square root transformation method was used for total neonates, total live neonates, and reproductive rate data. Growth data were not transformed because the data met the requirements of homogenous variance and normal distribution. Time to first brood data; however were analyzed using the non-parametric method.

The one-way and two-way ANOVAs were conducted with R-Program (Ver 3.1.1). Comparison for significant difference between the model coefficients of metal uptake was conducted using SAS (Ver 9.3.1). An effect with a $p < 0.05$ is considered significant.

RESULTS

Water chemistry

The average measured total and dissolved metal concentrations deviated approximately 0-15% from the nominal concentrations (except for the two lowest Zn concentrations) in the Cd-Zn mixtures and Zn alone treatments (Table 2, Supplemental Data). To be convenient, nominal concentrations are used to present the test treatments. However, measured dissolved Zn

concentrations in the test water of Zn alone and Cd-Zn mixture tests were used for modeling the relationship between water Zn concentration and body Zn and Cd concentrations (Fig. 11).

The average and standard deviation of hardness, alkalinity, pH, DO, and temperature for the Zn alone test were: 88.7 ± 7.18 mg/L as $CaCO_3$ (n=12), 61.5 ± 3.97 mg/L as $CaCO_3$ (n=12), 7.6 ± 0.16 (n=26), 7.3 ± 0.24 mg/L (n=26), and $22.6 \pm 0.40°C$ (n=26) respectively. Those same water quality parameters for the Cd-Zn mixture test were: 90.6 ± 4.52 mg/L as $CaCO_3$ (n=12), 60.4 ± 3.85 mg/L as $CaCO_3$ (n=12) 7.7 ± 0.15 (n=26), 7.7 ± 0.34 mg/L (n=26), and $22.3 \pm 0.45°C$ (n=26) respectively. Although organic carbon was not added into the test water, an average measurement of DOC concentrations of 4.44mg/L for Zn alone and 4.21 mg/L for Cd-Zn mixture tests were found (Table 3, Supplemental Data). The DOC was suspected as coming from the fed diet of *D. magna* (YCT and algae) and our DOC analysis on the YCT samples confirmed this suspicion. The average concentration of Cl^-, SO_4^{2-}, F^-, PO_4^{3-}, and Br^- of Zn alone and Cd-Zn mixture tests ranged from, 4.1 to 6.2 mg/L, 157 to 175mg/L, 0.6 to 0.7 mg/L, not detected to 0.26 mg/L, and not detected to 1.3 mg/L, respectively (Table 4, Supplemental Data). The average concentration range of Na^+, K^+, Ca^{2+}, and Mg^{2+} of Zn alone and Cd-Zn mixture tests were $25.6 – 33.4$, $2.1 – 3.0$, $12.4 – 14.4$, and $11.9 – 14.3$ mg/L, respectively.

Survival

The average 21-d cumulative percent mortality in the Cd-Zn mixtures containing 40, 80, or 120µg/L Zn were significantly lower in comparison to Cd alone ($p<0.01$) while the Cd-Zn mixtures containing 160 and 200µg/L Zn were significantly higher ($p<0.01$) (Fig. 1). There was no statistically significant difference in cumulative mortality between the Cd-Zn mixtures containing 10 and 20µg/L Zn and Cd alone. This indicates that when Zn is present at low concentrations, such as 10 and 20µg/L in a mixture with 1.5 µg/L Cd, mortality remains high and

the Cd effect on survival is still pronounced. However, as Zn concentrations increase in the mixtures (40, 80, and 120µg/L), mortality significantly decreases, reaching the lowest mortality at 80µg/L (Fig. 1). Therefore, at a sufficient concentration, Zn protects *D. magna* from the chronic toxicity of Cd, an evidence of a less-than-additive effect. However, given that the cumulative mortality was significantly different between Cd-Zn mixtures and Cd alone at higher Zn concentrations (i.e., 160, 200 µg/L), Zn accumulation likely exceeded the threshold for Zn-caused toxicity to *D. magna* and thereby caused most or all of the observed toxicity.

In comparing the cumulative percent mortality of Zn alone with its corresponding Cd-Zn mixture, the treatments containing 10, 20, or 40µg/L Zn were found to be significantly different ($p<0.05$) (Fig. 1). At these concentrations of Zn alone, there was no mortality or non-significant difference in mortality between the control and Zn alone treatments (Fig. 1). These results signify that the toxicity in those Cd-Zn mixtures would be due to Cd exposure. There was no statistically significant difference found in mortality between Zn alone and its corresponding Cd-Zn mixture at the higher Zn concentrations (80 to 200 µg/L). However, given that the cumulative mortality in the Zn alone treatments containing 80 and 120µg/L were not significantly different from the control; the toxicity observed in the corresponding Cd-Zn mixtures might also have been due to Cd exposure. At higher Zn concentrations (160 and 200µg/L), the toxicity observed in the Cd-Zn mixtures was likely due to Zn exposure because there was no significant difference in cumulative mortality between Zn alone and its corresponding Cd-Zn mixture.

Reproduction

Total neonates, live neonates, and reproductive rates: The effects of the Cd-Zn mixtures on the reproductive effort of *D. magna* are as follows. The total number of neonates ($p<0.05$) (Fig. 2) and the number of live neonates ($p<0.05$) (Fig. 3) reproduced by *D. magna* in the Cd-Zn

mixtures containing 40, 80, or 120µg/L Zn were significantly higher in comparison to Cd alone. Similarly, the overall reproductive rate ($p<0.05$) (Fig. 4), and the live neonate reproductive rate ($p<0.05$) (Fig. 5) were also significantly higher in the Cd-Zn mixtures containing 40, 80, or 120µg/L Zn than in Cd alone. No other Cd-Zn mixture had a significantly higher number of neonates or reproductive rates than that of Cd alone. These results suggest that Cd-Zn mixtures containing 10 and 20µg/L Zn are not sufficient to protect *D. magna* from Cd reproductive effect. *Daphnia magna* in those mixtures did not reproduce neither a higher number of total neonates nor a higher number of living neonates compared to Cd alone (Figs. 2, 3), thereby reflecting the reduction in their respective reproductive rates (Figs. 4, 5). However, increasing Zn concentrations in the mixtures (40, 80, and 120µg/L) inhibited Cd reproductive effects (evidence of a less-than-additive effect), which translated into a significantly higher number of both total neonates (Fig. 2) and live neonates (Fig. 3). These are represented in the higher overall reproductive rate shown in Figure 4 and the higher live neonate reproductive rate reflected in Figure 5. On the other hand, in mixtures containing higher Zn concentrations of 160 or 200µg/L, less-than-additive effects were not observed. Interestingly, only the Cd-Zn mixture containing 200µg/L Zn had significantly lower number of both total neonates ($p<0.05$) (Fig. 2), and living neonates ($p<0.05$) (Fig. 3) in comparison to Cd alone. Again at Zn concentrations of 160 and 200µg/L, Zn accumulation likely exceeded the threshold for Zn-caused toxicity to *D. magna* and thereby caused most or all of the observed toxicity. Similar to the survival results, the reproduction results also show less-than-additive effect of Cd-Zn mixtures when the Zn concentrations were sufficient but did not exceed the threshold for Zn-caused impairment.

The total number of neonates and the overall reproductive rate in Zn alone treatments containing 10, 20, 40, or 80µg/L showed no significant difference with the control (Figs. 2, 4).

Similarly, but not quite entirely, Zn alone concentrations of 10, 20, or 40µg/L were found to be non-toxic to *D. magna* reproduction of live neonates (Fig. 3) and Zn alone concentrations of 10, 20, 40, or 80µg/L were found to be non-toxic on the live neonate reproductive rate (Fig. 5). On the other hand, treatments containing higher Zn alone concentrations, such as 120, 160, or 200µg/L Zn were found to be toxic to both *D. magna's* reproduction of total neonates (Fig. 2) and live neonates (Fig. 3), since those treatments contained a significantly lower number of live neonates than the control. Additionally, the overall reproductive rate in Zn alone did not reveal toxic manifestations in any treatment, except at the highest Zn alone concentration (200µg/L) (Fig. 4). Likewise, in the live neonate reproductive rate, only the Zn alone treatments containing 120 and 200µg/L were found to be significantly affected (Fig. 5).

Sublethal Zn alone treatments containing 10, 20, or 40µg/L had a significantly higher number of total neonates (*p*<0.05) (Fig. 2), and a significantly higher overall reproductive rate (*p*<0.05) (Fig. 4) in comparison to their corresponding Cd-Zn mixtures. These results suggest that the reproductive effect observed in those Cd-Zn mixtures was due to Cd exposure. The Zn alone treatment containing 80µg/L did not exhibit a significantly higher number of total neonates (Fig. 2), nor a higher overall reproductive rate (Fig. 4) in comparison to its corresponding Cd-Zn mixture. In addition, the reproductive effect of Zn alone was not significantly different from the control. Thus the observed reproductive effects in this mixture were likely still due to Cd exposure. Higher concentrations of Zn alone treatments (e.g., 120 and 160µg/L Zn) were also found to have a significantly higher number of total neonates (Fig. 2) and a higher overall reproductive rate (Fig. 4) than their corresponding Cd-Zn mixtures. The one exception was the Zn alone treatment at 200µg/L. These results suggest that the observed reproductive effects in

the Cd-Zn mixtures containing 120 and 160µg/L in Figure 2 were due to a joint effect of both Cd and Zn, since Zn alone effects in those treatments were significantly lower than the control.

The results in the analysis of total live neonates (Fig. 3) and the live neonate reproductive rate (Fig. 5) were similar to the results obtained in the analysis of total neonates (Fig. 2) and the overall reproductive rate (Fig. 4). The only difference was that the Zn alone treatment containing 80µg/L were found to be significantly lower in comparison to the control when only live neonates were considered (Fig. 3). Moreover, since that same treatment was also found to contain a significantly higher number of live neonates than its corresponding Cd-Zn mixture (Fig. 3); the reproductive effect observed in the mixture was due to both Cd and Zn, a result that was not detected when both dead and live neonates were considered collectively (Fig. 2). Lastly, with respect to the live neonate reproductive rate (Fig. 5), the Zn alone treatment containing 120µg/L was significantly lower than the control, signifying that the reproductive effect observed in its corresponding Cd-Zn mixture was due to both Cd and Zn. Again, this was a result that was not detected when total neonates were considered (Fig. 4).

Total dead neonates and undeveloped embryos. The present study shows that except for the treatment containing 200µg/L Zn, other Cd-Zn mixtures had a significantly higher percentage of dead neonates than did the control ($p<0.05$) (Fig. 6). Moreover, the Cd-Zn mixtures containing 10, 20, or 40 µg/L Zn had a significantly higher percentage of dead neonates compared to the Cd alone treatment ($p<0.05$), whereas Cd-Zn mixtures containing higher Zn concentrations were not (Fig. 6). These results indicate that Zn did not protect neonate *D. magna* from Cd toxicity.

The percentage of dead neonates observed in the Cd-Zn mixtures containing 10, 20, or 40µg/L were significantly higher than the percentage of dead neonates observed in their corresponding Zn alone treatments ($p<0.05$) (Fig.6). This suggests that Cd plays an important

role in producing effects in those Cd-Zn mixtures. At higher Zn concentrations there was no significant difference in percentage of dead neonates observed in mixtures and their corresponding Zn alone treatments. This suggests that the role of Cd in producing effects in these mixtures is not important.

For the percentage of undeveloped embryos, all Cd-Zn mixtures and Cd alone were significantly different from the control with a *p-value* of less than 0.01 and 0.05, respectively. (Fig. 7). However, no Cd-Zn mixture held a significantly higher percentage of undeveloped embryos in comparison to Cd alone (Fig. 7).

Except for the 80 µg/L Zn, all Cd-Zn mixtures had a significantly higher percentage of undeveloped embryos in comparison to their corresponding Zn alone treatments ($p<0.05$) (Fig. 7). These results suggest that the effects observed in the Cd-Zn mixtures at low Zn concentrations (i.e., 10, 20, 40 µg/L Zn) were likely due to Cd exposure because their corresponding Zn alone treatments did not produce significant differences with the control (Fig. 7). At higher Zn concentrations, Zn alone treatments produced a significantly higher percentage of undeveloped embryos than did the control (Fig. 7). These results suggest that the effect observed in mixtures with higher Zn concentrations was due to both metals. Significant impaired reproduction was observed in the Cd-Zn mixture with 200µg/L Zn, and its corresponding Zn alone (Figs. 2, 3). Therefore, no undeveloped embryos were observed in those treatments.

The undeveloped embryos observed in the Cd-Zn mixtures were scanned for the presence of developmental obstructions and morphological alterations. A gravid adult *D. magna* from the laboratory culture was collected and dissected for embryos and used as a control. Compared to the control embryos, the embryos collected from the Cd-Zn mixtures revealed several morphological defects that either halted mitotic cell division (cleavage), or disrupted cellular

arrangement and organization which likely prevented the development of an embryo beyond stage 5 (Fig. 8H).

Time to first brood. The time to first brood for Cd alone was significantly earlier (9.25 days on average) in comparison to the control (11 days on average) ($p<0.01$) (Fig. 9). With exception to the Cd-Zn mixture containing 20µg/L Zn, the time to first brood of the other mixture treatments was significantly higher than that of Cd alone. In addition, there was no significant difference in the time to first brood between Zn alone and its corresponding Cd-Zn mixture except in the treatment containing 200 µg/L Zn, at which the time to first brood for the mixture treatment was also significantly higher than the control. These results indicate that the Cd-Zn mixture containing 200µg/L Zn increased the time to first brood (Fig. 9).

Growth effects

The dry weights of the surviving *D. magna* exposed to Cd-Zn mixtures containing 40, 80, or 120µg/L Zn were significantly higher than the dry weight of surviving organisms in Cd alone ($p<0.05$) (Fig. 10). Furthermore, the dry weight of the surviving organisms in the other Cd-Zn mixtures was not significantly higher in comparison to Cd alone (Fig. 10). Once more, these results imply that Cd-Zn mixtures containing 10 and 20µg/L Zn were not sufficient to induce protective effects from Zn to *D. magna*. However, Cd-Zn mixtures containing higher Zn concentrations (e.g., 40, 80, and 120µg/L Zn) strongly protected *D. magna* from Cd growth effect, reaching a peak at 80µg/L (evidence of less-than-additive effects). Conversely, a protective effect from Zn was not observed in the Cd-Zn mixture containing 160µg/L Zn. This result indicated that 160µg/L Zn exceeded the necessary concentration to protect *D. magna* from chronic Cd growth effect.

The dry weights of the daphnids in the Cd-Zn mixtures containing 10, 20, 40, 80, and 120µg/L Zn were significantly lower in comparison to the dry weight of their corresponding Zn alone treatments ($p<0.01$) (Fig. 10). These same Zn alone treatments were not significantly different from the control, except for the treatment containing 120µg/L Zn ($p<0.05$) (Fig. 10). Therefore, the growth effects observed in the Cd-Zn mixtures containing 10, 20, 40, and 80µg/L Zn were likely due to Cd exposure. However, the growth effect observed in the Cd-Zn mixture containing 120µg/L Zn was most likely due to both Cd and Zn, since the dry weight of the organisms in Zn alone treatment was significantly different from the control. At higher Zn concentration (160µg/L Zn), Zn became toxic to the organisms, and the effect observed in the Cd-Zn mixture was most likely due to Zn exposure.

Metal accumulation effects

The measured Cd concentrations in the body of surviving daphnids exposed to Cd alone or Cd-Zn mixtures were significantly higher than the measured Cd concentration in control organisms (Fig. 11). The results also showed that as Zn concentration increased in the exposure media of Zn alone and Cd-Zn mixture tests, Zn concentration gradually increased in the body of the surviving daphnids, fitting a power function (Fig. 11). Subsequently, a gradual decrease in body Cd concentrations followed a similar fitting power function (Fig. 11).

The results obtained from the body analysis of the surviving daphnids from Cd-Zn mixtures are consistent with the hypothesis that metals compete for binding sites on the biotic ligand. The organisms exposed to Cd alone accumulated significantly more Cd than organisms exposed to a mixture of Cd-Zn ($p<0.01$) (Fig 11). These results enforce a strong evidence of a competitive accumulation effect which results in a less-than-additive effect in Cd-Zn mixtures at Zn concentrations less than 120µg/L. It is also interesting that the coefficient of the uptake model

for Zn alone (75.556) was significantly higher than that for Cd-Zn mixture (52.898) (p=0.03). This indicates that body Zn concentrations of daphnids were higher in Zn alone treatments than in Cd-Zn mixtures. These results suggest an interference of Cd on Zn uptake.

DISCUSSION

In general, the results of the present study are consistent with other literature studies indicating a protective effect of Zn from Cd toxicity. For example, Meyer et al. [1] found a less-than-additive toxicity of Cd-Zn mixtures on *D. magna*. Cañizares-Villanueva et al. [19] reported reduced Cd toxicity on *D. magna* when Zn was present in a mixture after biological treatment with suspended cultures of *Chlorella vulgaris* in a 48 h assay (less-than-additive). Attar and Maly [20] also documented reduced Cd toxicity on *D. magna* after acute exposures to Cd-Zn mixtures. Other studies utilizing test organisms such as shrimp, flag fish, minnows, trout, and green algae have also reported less-than-additive toxicity between Cd and Zn mixtures [10, 21-25]. However, at high Zn concentrations in the mixtures, Zn became toxic to the organisms. The less-than-additive or joint toxic effect in Cd-Zn mixtures only occurred at certain concentrations of Zn and must be dependent on water quality characteristics that influence the bioavailability of metals, such as DOC, pH, and hardness. The discussion below is in the context of the water quality characteristics used in the present study.

Less-than-additive effect and competitive uptake

Overall, results of the present study indicate that at Zn concentrations less than 160 µg/L, the chronic effect of Cd-Zn mixture was less-than-additive. When Zn concentration is sufficient (not toxic when present alone), Zn appeared to protect the organisms from the chronic toxicity of Cd. The mechanisms of metal mixture toxicity is not clearly understood. It is believed that the less-than-additive toxicity of metal mixtures in aquatic organisms is due to the competitive

binding mechanism at the biotic ligand [1, 26]. Metals that have stronger affinity with the biotic ligand would occupy more binding sites, and therefore, be accumulated more in organisms than other metals that have less affinity with the biotic ligand. In the present study, we found that as Zn concentrations gradually increased in the exposure media of the Cd-Zn mixtures, body Zn concentration of *D. magna* subsequently increased, while the body Cd concentration decreased (Fig. 10). This finding supports the competitive binding mechanism of metals at the biotic ligand and agrees with the results published by Komjarova and Blust [27]. Benson et al. [28] also found similar results for acute toxicity with *D. magna* and Cd-Zn mixtures. The decrease in body Cd concentration in *D. magna* when increasing water Zn concentration explains the less-than-additive effect of the Cd-Zn mixtures. Typically, Zn produced protective effects against Cd toxicity to the organisms. The protective effect is more profound when Zn concentration is sufficient, for example mortality significantly decreased (Fig. 1) and reproduction (Figs. 2, 4) and growth (Fig. 9) significantly increased at Zn concentrations of 40, 80, and 120μg/L. At Zn concentrations of 10 or 20 μg/L, no significant protective effect of Zn against Cd toxicity was found. However, when Zn concentrations were greater than the necessary concentration to produce protective effects   (e.g., ≥160μg/L), Zn appeared to contribute to the toxicity.

Although body Cd concentration decreased with increasing water Zn concentration, it was still significantly higher than the body Cd concentration of the control organisms. This indicates that Zn did not completely block Cd binding to the biotic ligand. As a result, Cd still entered the organisms and produced certain levels of effects. For example, mortality in the mixtures did not completely decrease to 0% (mixture treatments 40, 80, 120 μg/L Zn, Fig. 1); reproduction rates of the mixtures were lower compared to the rates of Zn alone (mixture

treatments 40, 80, 120 µg/L Zn, Figs. 2-5); or more dead neonates and undeveloped eggs were found in the mixtures than in Zn alone treatments (i.e., 10, 20, 40 µg/L Zn, Figs. 6, 7).

Early reproduction, food consumption, and possible detoxification mechanism

It is interesting that *D. magna* in the Cd alone treatment produced neonates earlier than did the control *D. magna* (Fig. 9) (our coming unpublished results of Cd-Ni study showed similar effects). The fundamental question becomes what is the reason the organisms produced neonates earlier under the Cd alone condition? Yu and Wang [29] reported that up to 44-67% and 16-47% of selenium (Se) uptake by *D. magna* from the aqueous phase and dietary phase, respectively, were lost from the organisms via reproduction. The question of whether the transfer of a significant portion of accumulated Se to her offspring as a detoxification mechanism is still unanswered. In the present study, *D. magna* might have allocated energy towards earlier reproduction. In addition, when comparing the percentage of dead neonates produced in Zn alone and its corresponding Cd-Zn mixtures; the percentage of dead neonates produced in Cd-Zn mixtures containing 10, 20, or 40 µg/L Zn were significantly higher than those in Zn alone. Whether early reproduction with its higher percentage of dead neaonates is a mechanism to eliminate the adult's body Cd is still an unanswered question. Results of the present study did show a significant reduction in body Cd concentrations in these mixture treatments compared to Cd alone (Fig. 11). Further research is necessary to answer this question.

Our observations also showed that *D. magna* exposed to Cd alone did not consume much of the algae fed to them routinely. Consequently, lower reproduction rates and body weights in the Cd alone treatment compared to the control were observed in the present study. After 21 days of exposure, *D. magna* exposed to Cd alone were found to have experienced a 71.4% decrease in average body weights in comparison to control organisms (Fig. 10). Likewise, Bodar et al. [30]

exposed *D. magna* neonates to 1 and 5µg/L Cd and found that after 14 days of exposure, the body weights of *D. magna* in both Cd treatments had dropped to approximately 40% in comparison to the control. Bodar and colleagues [5] reported that Cd directly disrupted feeding behavior and digestive mechanisms, which resulted in altered metabolism, subsequently reducing food consumption. Another study by Jemec et al. [31] also found that Cd concentrations ≥0.656µg/L significantly affected *D. magna* reproduction and survival. Likewise, Elnabaraway et al. [32] demonstrated that a sublethal Cd concentration of 2.5µg/L significantly reduced fecundity of reproducing *D. magna* in as early as 14 days of exposure.

Effects on embryo development

The observation of undeveloped embryos in the present study indicates the effect of Cd and Zn on embryo development. The effect is more likely due to Cd since a higher number of undeveloped embryos was found in the Cd-Zn mixtures than in Zn alone (Fig. 7).

Fisher et al. [33] demonstrated that metals can directly adsorb onto developing embryos/neonates in the brood chamber and prevent embryo development. Another recent study investigating Cd embryotoxicity revealed that *D. magna* embryos experienced severe morphological defects after acute exposures to 60, 80, or 100µg/L Cd [34]. Neonates were observed to have many deformities which ranged from caudal spine malformations, to poorly developed carapaces, and no antennas or eyes [34]. Although the concentrations of Cd used in the study by Djekoun et al. [34] were between 1 and 2 orders of magnitude higher than the concentration of Cd used in the present study, it is clear that with the water chemistry of the present study, 1.5µg/L Cd, with chronic exposure is sufficient to induce embryonic deformities.

In examining embryonic morphological alterations, the study conducted by Mittmann et al. [35] was used as a reference (Fig. 8). Embryos collected from Cd alone, and the Cd-Zn

mixtures showed that the developing offspring experienced morphological deformities which translated either to the shutdown of mitotic cell division (early or late cleavage) (Fig. 8B, D); disruption of cellular arrangement and organization (gastrulation) (Fig. 8F, J), or complete prevention from further development beyond stage 5 (Fig. 8H) in comparison to the control embryos which displayed normal cleavage, a higher degree of cellular arrangement, organization, and differentiation (Fig. 8A, C, E, G, I).

Noticeably, the results further demonstrate that embryos exposed to Cd alone only underwent early cleavage, giving rise to a morula (Fig. 8B, while embryos exposed to the Cd-Zn mixture underwent late cleavage, giving rise to a blastula (Fig. 8D), gastrulation (Fig. 8F, J), or development to stage 5 (Fig. 8H). Although some embryos were able to form a blastula and initiate gastrulation, the blastomeres were totally disorganized or deformed, and conglomerated into a blob of cells (Fig. 8F, J). This suggests that at some degree the blastomeres lost their ability to successfully communicate, and were unable to faithfully arrange and organize themselves. The effects of Cd on the morphological development of *D. magna* could be due to the disruption of biochemical signals that are essential to the successful development of *D. magna* embryos or it could be acting as a genotoxic agent that leads to the premature death of the developing embryos. Filipic and Hei [36] reported that Cd has high mutagenic activity since it predominantly induces large deletion mutations. In addition, Cd is able to interact with reactive oxygen species (ROS), disrupting cellular signaling and interfering with DNA repair [36].

Since Cd and Zn inhibit Ca uptake, these metals might have disrupted calcium homeostasis, and altered Ca-dependent cellular signaling. Cytoplasmic Ca signaling is known to regulate genes associated with differentiation, growth, and apoptosis, and disruption of those signaling pathways would lead to deleterious effects, including DNA impairment, transcriptional

abnormalities, and malignant cellular growths [37]. Cadmium is able to inappropriately activate Protein Kinase C (PKC) at nM concentrations, but also reversibly, inhibit its activity at µM concentrations [38, 39]. In addition, Cd can also replace the Ca^{2+} ion on Ca-dependent signaling proteins and disrupt the normal functions of those proteins [40]. For example, calmodulin, another Ca dependent signaling protein, is able to strongly bind to, modify, and interact with many target proteins, including kinases and phosphatases inducing several cellular signaling pathways [41]. These cellular effects are central to the toxic mechanisms of Cd.

It is interesting to mention that at concentrations of 40, 80, 120 µg/L, Zn protected adult *D. magna* from Cd toxicity (Fig. 1). The protective effect by a metal against toxic effect by another metal to aquatic organisms is attributed to a competitive binding mechanism at the biotic ligand [1, 26]. However, there was no significant reduction in the percentage of undeveloped embryos in the mixtures compared to Cd alone (Fig. 7). This indicates that Zn did not have a protect effect on embryonic development in the mixtures. On the other hand, the percentage of undeveloped embryos were higher in the mixtures than their corresponding Zn alone treatment, signifying that Cd still plays a major role in affecting embryonic development in the mixtures. The effect on embryonic development could be due to accumulated Zn and Cd in *D. magna*. When looking at metal uptake at these water Zn concentrations, we found that body Zn concentration increased while body Cd concentrations in adult *D. magna* decreased (Fig. 11). Nevertheless, even with higher concentration of Zn accumulation in the body, low concentration of Cd still had a negative influence on embryonic development. This raises a question of whether competitive binding plays a major role in modifying impairment of embryonic development.

CONCLUSIONS AND SUGGESTIONS

Given the water chemistry parameters used in the present study, with optimal water Zn concentrations ranging from 40 to 120 µg/L; Zn protected *D. magna* from the chronic toxicity of Cd, resulting in less-than-additive effects on survival, growth and reproduction. Zinc concentrations that are outside of the optimal range can either be insufficient to protect the organisms from Cd toxicity or join the toxic effects of Cd. In general, when water Zn concentration increased, body Zn concentration increased and body Cd concentrations decreased. This explains the protective effect of Zn from Cd toxicity due to competitive binding mechanism between Cd and Zn at the biotic ligand. However, Cd affected embryonic development of *D. magna* regardless of the presence of Zn in the water or in the organisms. This embryonic effect is not explained by the competitive binding mechanism at the biotic ligand and raises a question of whether competitive binding plays a major role in modifying impairment of embryonic development? More studies should be conducted to characterize the interactive mechanisms between metals at the cellular level in organisms. Results of the present study are useful for development of metal mixture BLM and environmental quality guidelines.

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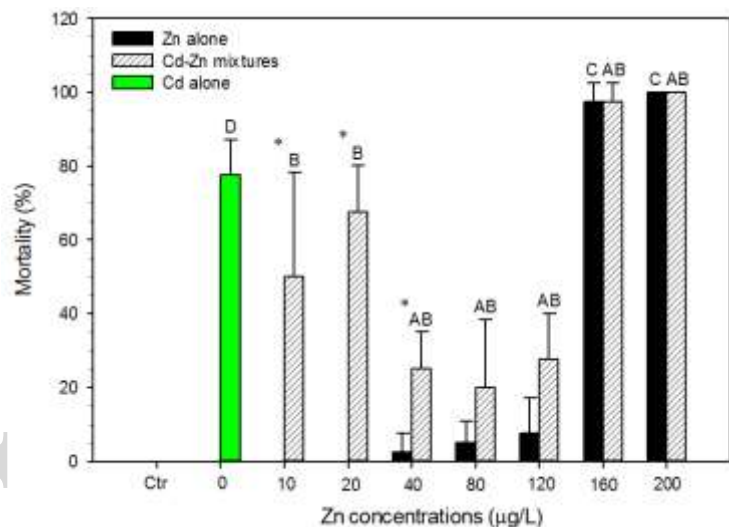
Fig. 1. 21-d cumulative mortality of *D. magna* due to exposure to Cd alone, Zn alone, and Cd-Zn mixtures containing constant Cd concentration of 1.5µg/L and varied Zn concentrations. A: significant differences between Cd alone and Cd-Zn mixtures ($p<0.01$). B: significant differences between control and Cd-Zn mixtures ($p<0.001$). C: significant differences between control and Zn alone ($p<0.001$). D: significant difference between control and Cd alone ($p<0.001$). *significant differences between Zn alone and Cd-Zn mixtures ($p<0.05$).
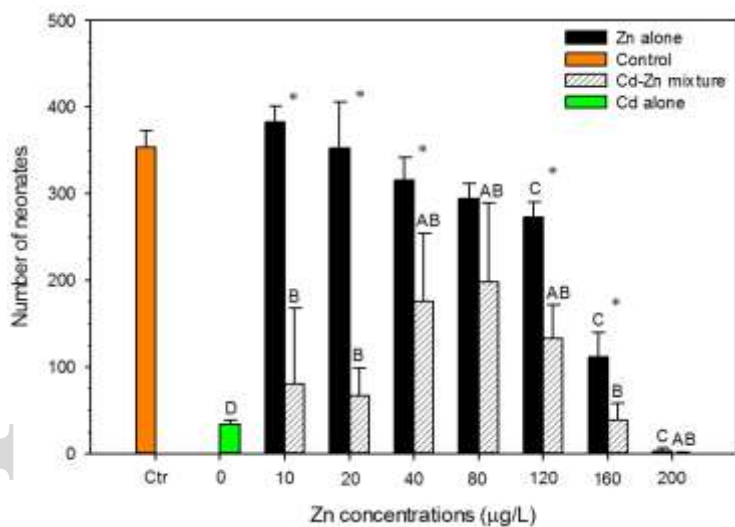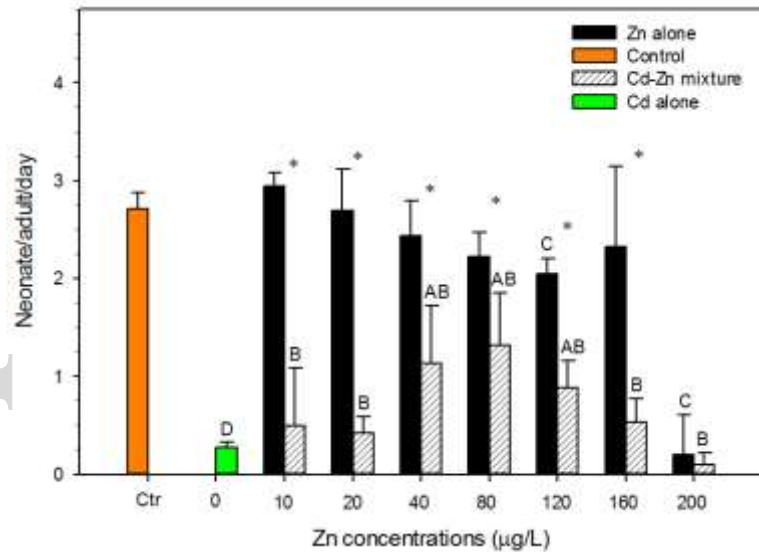

Fig. 2. Total neonates (dead and live) reproduced over 21-d of exposure to Cd alone, Zn alone, and Cd-Zn mixtures containing constant Cd concentration of 1.5µg/L and varied Zn concentrations. A: significant differences between Cd alone and Cd-Zn mixtures ($p<0.05$). B: significant differences between control and Cd-Zn mixtures ($p<0.05$). C: significant differences between control and Zn alone ($p<0.05$). D: significant difference between control and Cd alone ($p<0.001$).*significant differences between Zn alone and Cd-Zn mixtures ($p<0.05$).

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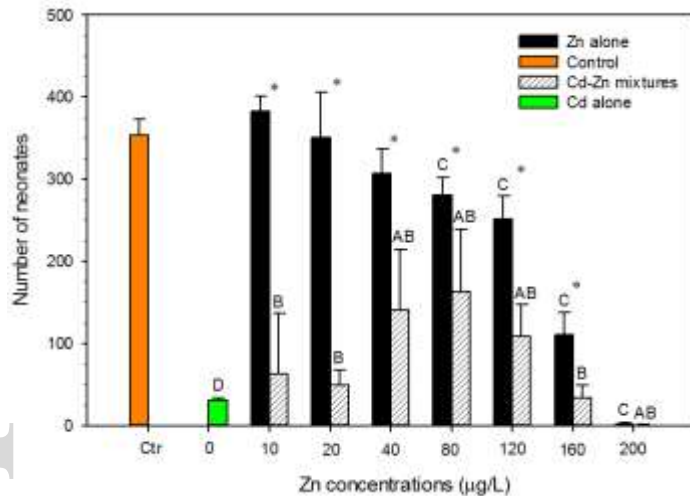
Fig. 3. Total live neonates reproduced over 21-d of exposure to Cd alone, Zn alone, and Cd-Zn mixtures containing constant Cd concentration of 1.5µg/L and varied Zn concentrations. A: significant differences between Cd alone and Cd-Zn mixtures ($p<0.05$). B: significant differences between control and Cd-Zn mixtures ($p<0.01$). C: significant differences between control and Zn alone ($p<0.05$). D: significant difference between control and Cd alone ($p<0.001$). *significant differences between Zn alone and Cd-Zn mixtures ($p<0.01$).
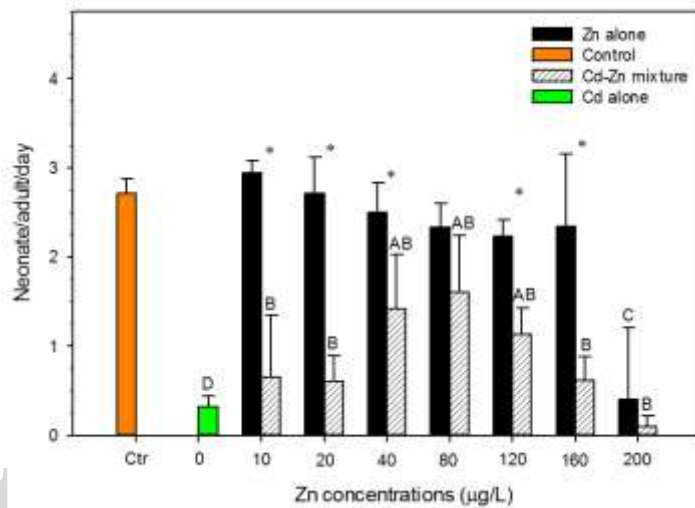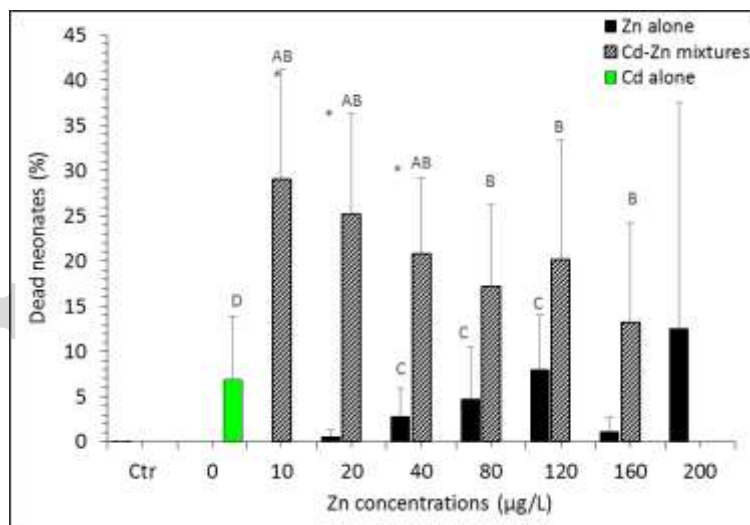

Fig. 4. Overall reproductive rate (dead + live neonates) of *D. magna* when exposed to Cd alone, Zn alone, and Cd-Zn mixtures containing constant Cd concentration of 1.5µg/L and varied Zn concentrations. A: significant differences between Cd alone and Cd-Zn mixtures ($p<0.05$). B: significant differences between control and Cd-Zn mixtures ($p<0.05$). C: significant differences between control and Zn alone ($p<0.05$). D: significant difference between control and Cd alone ($p<0.001$). *significant differences between Zn alone and Cd-Zn mixtures ($p<0.05$).
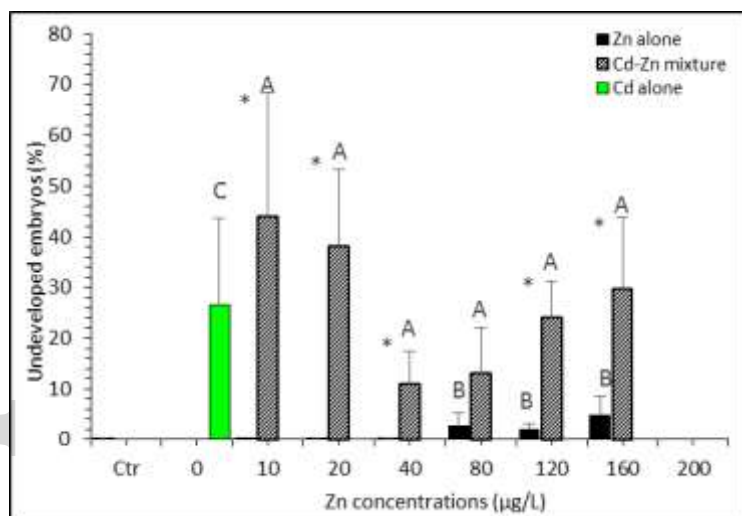
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Fig. 5. Reproductive rate (live neonates) of *D. magna* when exposed to Cd alone, Zn alone, and Cd-Zn mixtures containing constant Cd concentration of 1.5μg/L and varied Zn concentrations. A: significant differences between Cd alone and Cd-Zn mixtures (*p*<0.05). B: significant differences between control and Cd-Zn mixtures (*p*<0.01). C: significant differences between control and Zn alone (*p*<0.05). D: significant difference between control and Cd alone (*p*<0.001). *significant differences between Zn alone and Cd-Zn mixtures (*p*<0.05).
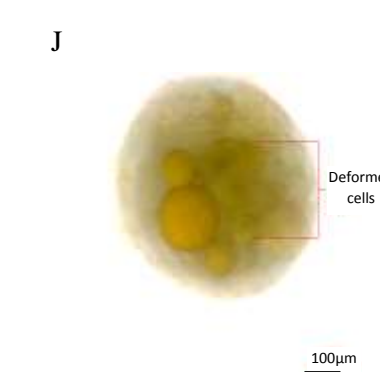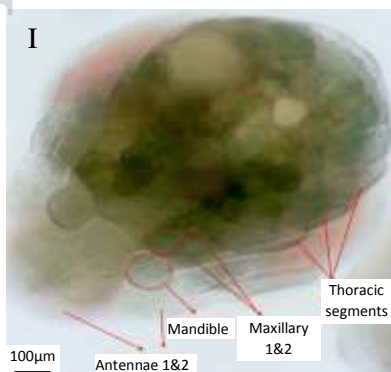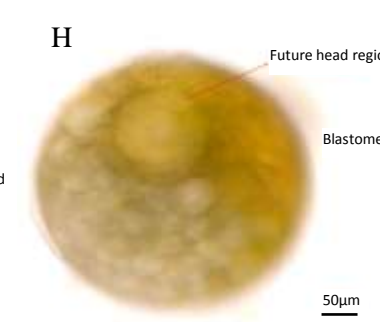

Fig. 6. Percent of dead neonates reproduced over 21-d of exposure to Cd alone, Zn alone, and Cd-Zn mixtures containing constant Cd concentration of 1.5μg/L and varied Zn concentrations. A: significant differences between Cd alone and Cd-Zn mixtures (*p*<0.05). B: significant differences between control and Cd-Zn mixtures (*p*<0.05). C: significant differences between control and Zn alone (*p*<0.05). D: significant difference between control and Cd alone (*p*<0.05). *significant differences between Zn alone and Cd-Zn mixtures (*p*<0.05).
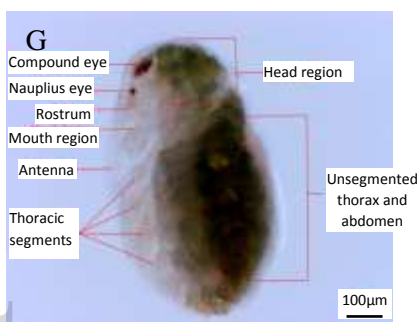
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Fig. 7. Percent of undeveloped embryos observed over 21-d of exposure to Cd alone, Zn alone, and Cd-Zn mixtures containing constant Cd concentration of 1.5µg/L and varied Zn concentrations. A: significant differences between control and Cd-Zn mixtures ($p<0.01$). B: significant differences between control and Zn alone ($p<0.05$). C: significant difference between control and Cd alone ($p<0.05$). *significant differences between Zn alone and Cd-Zn mixtures ($p<0.05$).

4

A — Blastomere — 100µm

B — Blastomere — 100µm

C — Future head region — 100µm

D — Blastomere — 50µm

E — Head region — Blastomere — 100µm — 100µm

F — Disorganized cells — 100µm

G — Compound eye — Nauplius eye — Rostrum — Mouth region — Antenna — Thoracic segments — Head region — Unsegmented thorax and abdomen — 100µm

H — Future head region — Blastomere — 50µm

I — Mandible — Maxillary 1&2 — Thoracic segments — Antennae 1&2 — 100µm

J — Deformed cells — 100µm

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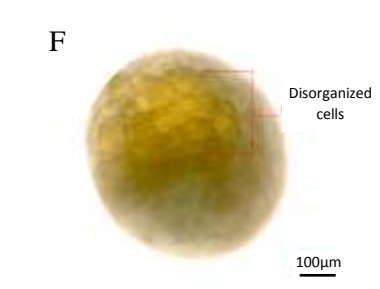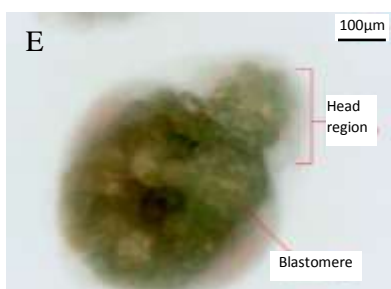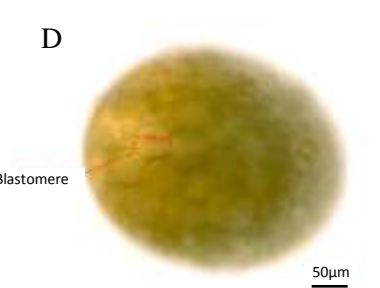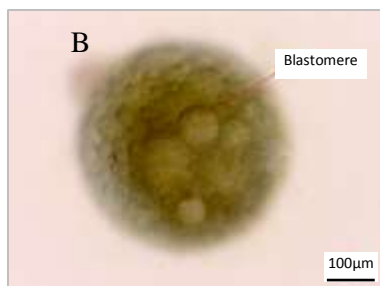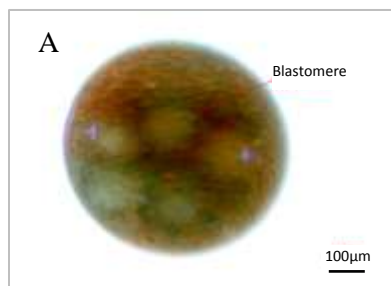
Fig. 8. Embryo development of *D. magna* over 10 stages.

A: Control embryo in stage 2 of development (early cleavage), B: Embryo in stage 2 of development (early cleavage) of Cd alone, C: Control embryo in stage 7.3 of development (Postnaupliar segments, ventral view), D: Embryo in stage 2 of development (late cleavage) for Cd-Zn mixture with 0µg/L Zn, E: Control embryo in stage 9 of development (appearance of compound and nauplius eye, dorsal view), F: Embryo in stage 3 of development (gastrulation) for Cd-Zn mixture at 20µg/L Zn, G: Control embryo in stage 9 of development (lateral view), H: Embryo in stage 5 of development (head formation, anterodorsal view) for Cd-Zn mixture at 160µg/L Zn. I: Control embryo in stage 10 of development (hook-shaped abdomen, ventral view), J: Embryo in stage 3 of development (gastrulation, ventral view) for Cd-Zn mixture at 40µg/L Zn.
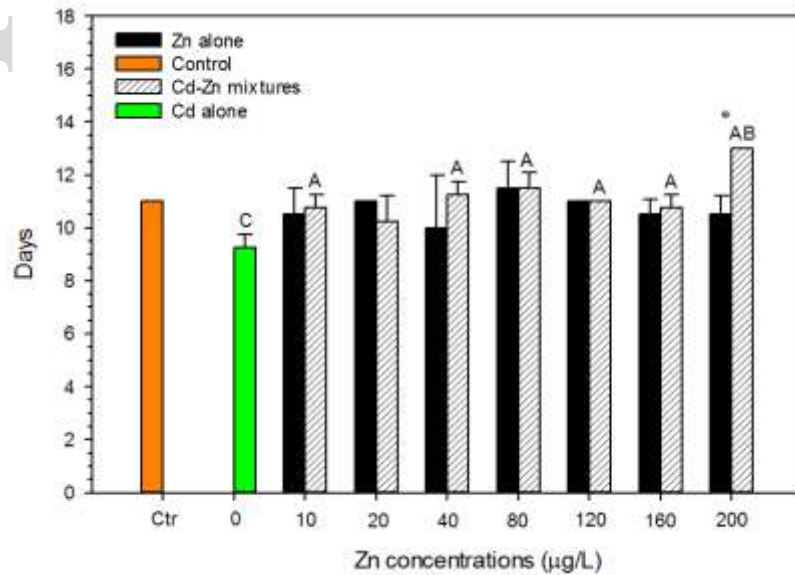

Fig. 9. Time to first brood over 21-d of exposure to Cd alone, Zn alone, and Cd-Zn mixtures containing constant Cd concentration of 1.5µg/L and varied Zn concentrations. A: significant differences between Cd alone and Cd-Zn mixtures ($p<0.01$). B: significant differences between control and Cd-Zn mixtures ($p<0.01$). C-significant differences between control and Cd alone ($p<0.01$). *significant differences between Zn alone and Cd-Zn mixtures ($p<0.01$).
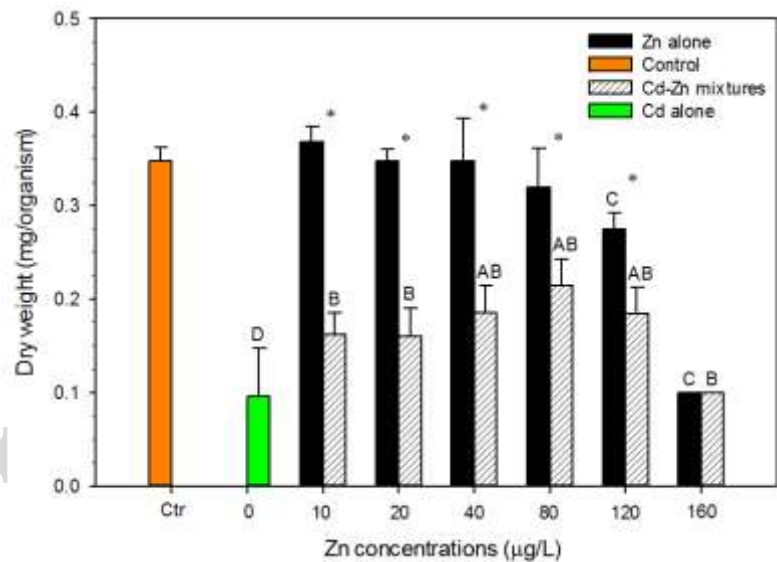
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Fig. 10. Dry weight of surviving adults at test termination. A: significant differences between Cd alone and Cd-Zn mixtures ($p<0.05$). B: significant differences between control and Cd-Zn mixtures ($p<0.05$). C: significant differences between control and Zn alone ($p<0.05$). D: significant difference between control and Cd alone ($p<0.001$). *significant differences between Zn alone and Cd-Zn mixtures ($p<0.01$).
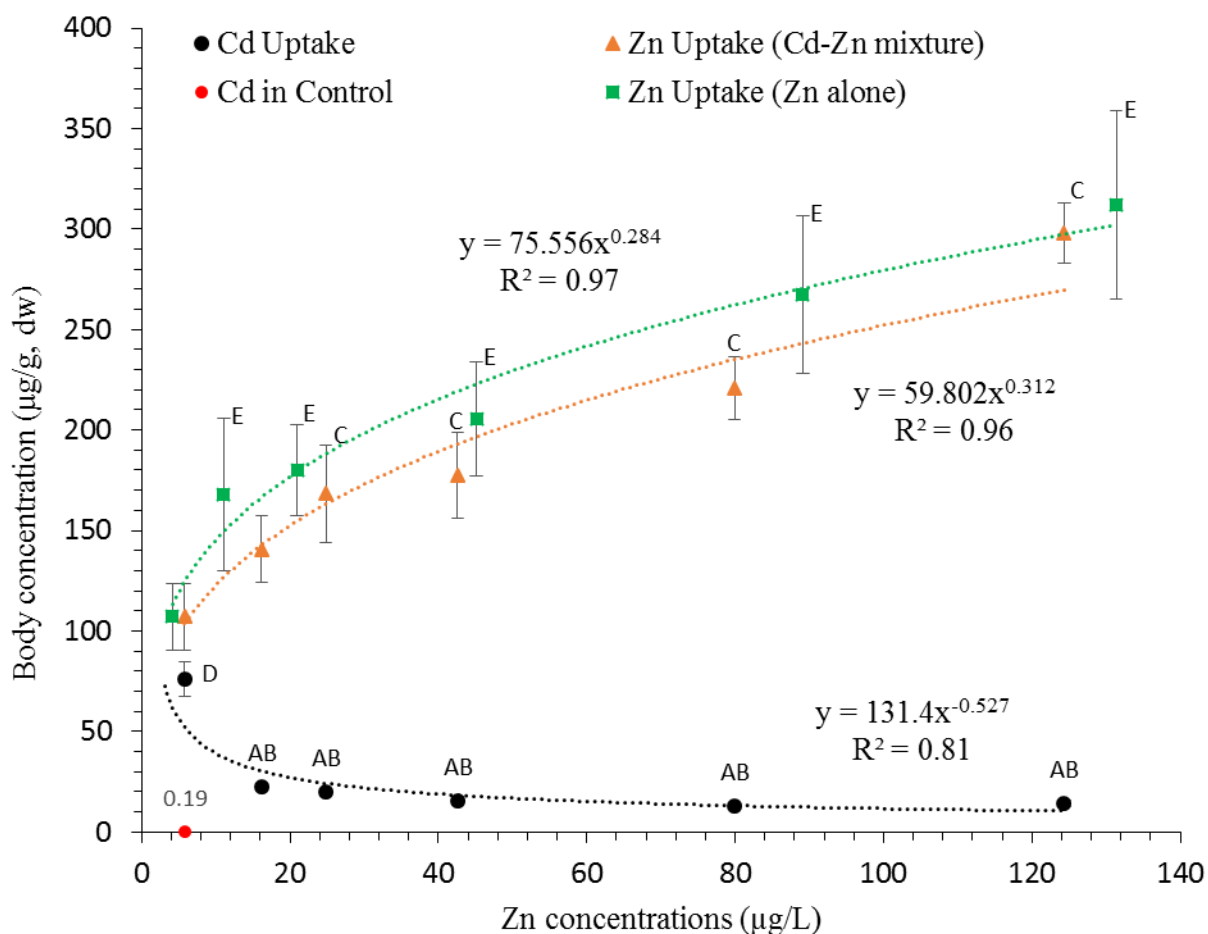
Fig. 11. Metal concentrations in the body of surviving daphnids exposed to Zn alone and Cd-Zn mixture over 21 days. A: significant differences between Cd alone and Cd-Zn mixtures ($p<0.05$). B: significant differences between control and Cd-Zn mixtures ($p<0.05$). C: significant differences between control and Cd-Zn mixtures ($p<0.05$). D: significant difference between control and Cd alone ($p<0.001$). E: significant differences between Zn alone and control.