Acute and chronic toxicity of mixtures of bisphenol A and trace metals (Cd and Pb) to micro-crustacean, *Daphnia magna*

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**Abstract.** The occurrence of plastic additives and their ecological impacts have attracted much attention in recent years globally. Among plastic additives, the trace metals (e.g., Cd, Pb) are widely used as color pigments and stabilizers, whereas bisphenol A (BPA) is added to enhance the desired physical characteristics of plastic products. However, these additives can easily leach out of plastic materials and enter the aquatic environment causing risks to aquatic ecosystems. Although the toxicity of a single additive on various aquatic organisms has been studied, the responses of zooplankton exposed to the mixed plastic additives have not been fully understood. Therefore, this study aims to evaluate the effects of the binary mixtures (BPA+Cd, BPA+Pb) and trinary mixture (BPA+Pb+Cd) at the metal concentrations of 5 µg/L and BPA level of 50 µg/L on the life history traits and food feeding rate of the freshwater micro-crustacean, *Daphnia magna*. The results showed exposures to these mixtures for 24h could significantly enhance the food feeding rate of *D. magna* from 2.5 – 5.8 times higher than the control. The survival rate was decreased from 50 – 90% in the organisms exposed to these mixtures after 18 incubated days. We found a synergistic effect of BPA+Pb but an antagonistic effect of BPA+Pb+Cd on the survivorship of *D. magna*. Similarly, the organisms in the exposures delayed their maturity age and reduced their reproduction. The potent impact order of the mixtures on *D. magna* was BPA+Cd > BPA+Pb+Cd > BPA+Pb. Our results evidenced the adverse effects of plastic additive mixtures on aquatic organisms. Therefore, the use and disposal of plastic materials and plastic additives should be paid more attention to protect the environment, ecosystem, and human health. Moreover, our findings proved that the toxicity of multi-contaminants on organisms could be unpredictable even the toxicity of a single contaminant is known.

**Keywords.** plastic additives, cladoceran, antagonistic effects, synergistic effects

1. **Introduction**

In the industry of plastic manufacture various chemicals so called plastic additives (e.g., bisphenol A, trace metals, phthalate, among others) are used to form different beneficial characteristics of plastic products such as flexibility, thermal and corrosive resistance, hardening or colors [1]. Among the plastic additives, bisphenol A (BPA) has been widely used in various plastic products such as polyvinyl chloride (PVC), polypropylene, and polyethylene [1,2]. The global consumption of BPA has rapidly increased in recent years reaching approximately 8 million tons in 2016 [3]. Obviously, most plastic additives including BPA are not chemically but physically bound to the polymers, therefore, they can be easily released out of plastic materials entering the aquatic environment then available for organisms [1]. The release of BPA and other additives (e.g., trace metals, phthalate) from plastic products has been reported
in numerous studies [4, 5, 6]. Also BPA presence in water bodies can be due to the discharges from industrial manufacturing [2]. As a result, BPA is among the plasticizers frequently detected in aquatic environment, in which the highest concentrations found in surface water and in wastewater were > 90 µg/ L and 370 µg/ L, respectively [3]. In Vietnam, the results from the study of Tam et al. [7] indicated that the concentration of BPA in many canals could reach 30 ng/ L, but Vietnam has been listed as among the four countries in the world discharging mismanaged plastic waste into the ocean [8]. Therefore, it could be predicted that the concentration of BPA in water bodies in Vietnam may continuously increase in the next few years. As an endocrine disrupting compound, BPA could not only cause the disruption of intracellular systems but also interfere with the functions of hormones in various organisms that could be considered as a potential risk for aquatic ecosystems and human health [1, 2].

On the other hand, Cd and Pb are among the trace metals frequently detected in aquatic environment at high levels [9, 10]. Although the occurrence of Pb and Cd in the environment is partly due to natural processes, the discharges from anthropogenic activities are mainly responsible for the contamination of these metals in water bodies [11, 12]. Cd is commonly used in nickel-cadmium battery production and special alloys to avoid corrosion due to its non-corrosive properties while Pb plays an important role in battery manufacturing, paints, and gasoline [11, 12]. Moreover, these metals are also used as stabilizers in plastic production, especially PVC [11, 12]. Due to the wide application in many industrial processes, the concentrations of these metals in water bodies have been frequently detected at high levels [9, 10]. In surface water, the concentrations of Cd and Pb could be up to 190 and 430 µg/L, respectively [10], but those could reach higher 4.5 mg/L and 17 mg/L, respectively, in industrial areas [9]. On the other hand, the previous study of Dang et al. [13] recorded the highest concentration of Cd and Pb in the Red River, which is among the main rivers in Vietnam could reach approximately 50 and 480 µg/L, respectively, while the safety concentration of these metals according to the technical regulation for surface water quality of Vietnam are 5 and 20 µg/L, respectively. Seriously, both Cd and Pb are non-essential elements to living things, but they can be highly toxic to aquatic organisms even at a low concentration [14]. Pb has been known as a neurotoxic chemical and exposure to this metal could cause the increase of oxidative stress leading to numerous effects on the behavior, nervous and respiratory systems, reproduction, and enzymatic functions of living things [15, 16]. On the other hand, Cd has been reported to cause detrimental influences on enzymatic systems and induce oxidative stress in organisms [14].

In aquatic ecosystems, zooplankton (e.g., *Daphnia magna*) play a vital role as they have a central position in the food web connecting major primary producers (phytoplankton) with other organisms at higher trophic levels (fish, shrimps) [17]. Besides, they not only have high sensitivity to toxic substances but are also easily maintained in the laboratory conditions. Therefore, *D. magna* was strongly suggested as a model organism in toxicological investigations [18, 19, 20]. There have been numerous studies showed the singly detrimental effects of BPA and the metals Pb and Cd, on *D. magna*. For instance, BPA could cause lethal effects on *D. magna* with the median 48h-EC50 value of 8.4 mg/L while those of Cd and Pb have been reported to be 21 and 695 µg/L, respectively [21, 22]. At sub-lethal concentrations, BPA could induce genotoxicity, the alteration in lipid peroxidation levels and enzymatic activities in *D. magna* [23, 24, 25]. Nagato et al. [26] showed a significant change in the metabolisms of *D. magna* exposed to BPA while the adverse influences of BPA on the life history traits of *D. magna*, including survival rate, reproduction, maturation, and growth, have also been reported in numerous studies [3, 23, 24]. Similarly, exposures to Cd and Pb could decline the survivorship and inhibit the reproductive performance of *D. magna* [27, 28, 29, 30, 31]. Moreover, Cd has been reported to cause a decrease in the assimilation rate and biochemical effects in *D. magna* [32] while exposure to Pb could also result in an alteration in the content of enzymes and hemoglobin leading to the impairment in growth and reproduction of the organisms [27].
Although the single effects of BPA, Cd and Pb on D. magna have been well documented, the combined toxicity of these chemicals on this organism is understudied. It is important to note that toxicity to organisms in aquatic environments could not result from a single chemical but due to the mixtures of various pollutants. Moreover, previous studies have indicated that the combined effects of the pollutant mixtures on organisms could differ from that of a single pollutant [33, 34, 35]. For instance, Meng et al. indicated the synergistic toxicity of the mixture of five metals (Hg, Cd, Cu, Pb and Cr) on D. magna [36]. Similarly, Cooper et al. found a higher toxicity of the binary and ternary mixtures of Pb, Cu, and Zn on reproduction of Ceriodaphnia dubia compared to the single effects of each metal [35]. Additionally, the single effects of Cd, Cr and Cu on the marine copepod Tsbe holothuriae could be less toxic than the joint effects of the mixtures of these metals [33]. The different responses of the freshwater micro-crustacean C. dubia in the exposures to the mixtures of metals (Cu and Zn) and organophosphate compared to those exposed to these pollutants individually could be another typical example [34]. To the best of our knowledge, there is gap on the mixture toxicity of BPA and non-essential metals on freshwater zooplankton upon chronic exposures. Therefore, this study aimed to assess the joint toxicity of BPA and the two trace metals (Cd and Pb) on the life history traits of D. magna over a period of 18 days. Besides, the food feeding rate of D. magna in an acute exposure to the mixtures of these chemicals were also investigated in our study.

2. Materials and methods

2.1. The test organisms and chemicals for the experiment

The micro-crustacean, Daphnia magna was used for the acute and chronic experiments. The organisms were obtained from MicroBio Test (Belgium) and have been maintained in the ISO medium [37] for many generations under laboratory conditions of 24 ± 1 °C, light intensity < 1000 Lux, and 14h light followed by 10h dark regime APHA [20]. The organisms were fed with the mixture of the green alga Scenedesmus protuberans and YTC (yeast, cerophyl, and trout chow digestion) [38]. The plastic additive Bisphenol A (purification of 99%) was purchased from Aldrich Sigma. The BPA was dissolved in acetone (Merck) at the concentration of 1000 mg/L which was used as the stock solution before spiked into the ISO medium to have a test concentration for experiments. Meanwhile, both the metal solutions Pb(NO₃)₂ and Cd(NO₃)₂ at the concentration of 1000 mg/L (ICP/MS standard analysis; Merck) were used as stock solutions for the experiments. All the stock solutions were stored at a temperature of 4 °C before the experiment.

2.2. Experimental setup

2.2.1. Acute exposure on the food feeding rate. The acute experiment was performed to assess the effects of the mixtures of BPA and metals on the food filtering activity of D. magna. The food filtering test was designed according to Jesus et al. with minor modifications [39]. Briefly, the test was initiated with D. magna of four days old. The organisms were exposed to (1) a mixture of BPA (50 µg/L) and Pb (5 µg/L), abbreviated as BPA+Pb; (2) a mixture of BPA (50 µg/L) and Cd (5 µg/L), abbreviated as BPA+Cd; and (3) a mixture of BPA (50 µg/L), Pb (5 µg/L) and Cd (5 µg/L), abbreviated as BPA+Pb+Cd. Besides, a positive control was also prepared in parallel with the exposures by culturing the organisms in the ISO medium without BPA and the metals. In each treatment (positive control or exposures), 10 organisms (n = 10) were used and each organism was individually raised in a 50 mL glass beaker containing 40 mL ISO medium. In each beaker, one organism was fed with a similar initial amount of S. protuberans (52,050±2,158 cells/mL). In addition, to confirm if the S. protuberans could grow in ISO medium (containing no nitrogen and phosphorus) or not, we conducted a negative control with ten beakers (n = 10) in which each contained 40 mL ISO medium and a similar initial algal abundance with other exposures but no D. magna. The experiment was conducted in the laboratory conditions (temperature, light intensity, and light: dark regime) as mentioned above. After 24h the algal abundance in each beaker was counted [40] with a Sedgewick Rafter counting chamber (Pyser SGI, England) under a microscope (Optika B150, Italy). Then the abundance of S. protuberans in each beaker
at the start and after 24h was used to assess the food feeding rate (F) of the organisms in control and exposures.

2.2.2. Chronic exposure on life history traits. The chronic experiments to evaluate the toxicity of the mixture of BPA and metals were performed according to APHA with minor modifications [20]. Prior to the experiments, nearly 50 mother D. magna were randomly selected and then incubated in a 2 L glass beaker containing 1.5 L of ISO medium. Neonates (< 24h old) from the second brood of mother D. magna in the beaker were randomly collected and used for the chronic test [37]. Four treatments including one control and three exposures were prepared including BPA+Pb, BPA+Cd, and BPA+Pb+Cd (as mentioned in food feeding rate experiment above). The BPA and metal concentrations were chosen basing on the concentration of BPA ever detected in the environment and the safe concentrations of Pb and Cd regulated in the Vietnam Technical Regulation for surface water safety (QCVN 08-MT:2015/BTNMT). For each treatment, 2 neonates were incubated together in a 50 mL glass beaker containing 40 mL ISO medium. There were 15 replicates in each treatment (n=15). Besides, the control was conducted in parallel with the exposures by raising organisms in the ISO medium free pollutants (BPA, Cd, Pb). The D. magna in each beaker were fed with S. protuberans and YTC [38] during an experimental period of 18 days under the laboratory conditions as mentioned above. The medium and food in each beaker was totally renewed three times per week while the life history traits of D. magna including survival, total neonates, and maturation age, were carefully recorded daily.

2.3. Data treatment. The abundance of S. protuberans in the negative control at the start and the end of the acute test was 52,050±2,158 cells/mL and 49,360±2,797 cells/mL, respectively. This meant the abundance of S. protuberans in the negative control did not increase after 24h in ISO medium as we expected. Therefore, the food feeding rate (F, cells/hour) of one D. magna was calculated as the equation of F = V(C_0 – C_t)/t [39], where:

V, volume of the test solution (40 mL in this study);
C_0, abundance of S. protuberans (cells/mL) at the start of experiment;
C_t, abundance of S. protuberans (cells/mL) when the test terminated;
t, exposure time (24 hours in this study).

Sigma Plot Version 12.0 was used for data analyses. The Kruskal-Wallis tests were applied to calculate the statistically significant difference in the food feeding rate and the maturity age of D. magna between the control and exposures. A gap of more than 20% in the survival proportion of D. magna in the exposures was considered as a significant difference [20].

3. Results and discussion

3.1. Physical and chemical characteristics of the culturing medium

The pH and dissolved oxygen (DO) concentrations of the culturing medium in each treatment were determined at the beginning and before the medium renewal. The pH values of the medium in the control and exposures did not significantly alter during the time of the test and ranged from 7.25 to 7.44 (Metrohm 744) while the DO ranged from 6.60 to 7.28 mg/L (Hanna HI9146). Besides, the hardness and alkalinity of the test medium were analyzed by titration method according to APHA [17] and their values were from 288 - 341 mg CaCO_3/L and 32 - 50 mg CaCO_3/L, respectively (Table 1). The hardness and alkalinity values revealed that the test media was a medium hardness water [38] whereas the pH and DO values of the media in all the exposures including the control met the standard requirement of APHA [17] for culturing the D. magna.
Table 1. The physical and chemical characteristics of the test medium

| Exposures          | pH       | Temperature (ºC) | DO (mg/L) | Hardness (mg CaCO₃/L) | Alkalinity (mg CaCO₃/L) |
|--------------------|----------|------------------|-----------|------------------------|-------------------------|
| Control            | 7.25–7.43| 23–24            | 7.28–7.31 | 288–271                | 44–50                   |
| BPA+Pb             | 7.43–7.63| 23–24            | 7.06–7.14 | 354–296                | 32–42                   |
| BPA+Cd             | 7.41–7.44| 23–24            | 6.38–6.98 | 337–300                | 32–42                   |
| BPA+Pb+Cd          | 7.43–7.44| 23–24            | 6.60–6.63 | 341–300                | 33–41                   |

3.2. The effects on the food feeding rate of Daphnia magna

The results of food feeding rate by *D. magna* were showed in the Table 2. During the experimental period, one *D. magna* in the control fed the *S. protuberans* at a rate of 4,870±2,408 cells/h. However, the feeding rate in the BPA+Pb, BPA+Cd, and BPA+Pb+Cd respectively was 11,967±1,965, 28,043±4,048, and 13,667±7,182 cells/h which was around 2.5, 5.0, and 2.8 fold higher than the control (Table 2). Therefore, the mixtures of BPA and trace metals (Pb, Cd) made the organisms significantly consume more food (*S. protuberans*). Besides, the food feeding rate of *D. magna* in the treatment BPA+Cd was significantly higher than that in the other exposures (p < 0.001; Table 2).

Table 2. The food feeding rate (F) of *Daphnia magna* during 24h of experiment. Different letters (a, b, c) indicated the significant difference of the food feeding rate among the treatments (p < 0.001) according to the Kruskal-Wallis test.

|                | Control          | BPA+Pb           | BPA+Cd           | PBA+Pb+Cd         |
|----------------|------------------|------------------|------------------|-------------------|
| F (mean ± SD; cells/h) | 4,870±2,408(a) | 11,967±1,965(b) | 28,043±4,048(c) | 13,667±7,182(b)  |
| Proportion to the control | 100%             | 250%             | 580%             | 280%              |

Negative effects of singly BPA or trace metals on food consumption of *Daphnia* were reported. The food assimilation rate of *Daphnia* spp. exposed to Cd (from 10 to 100 µg/L) was reduced which could be the consequences of feeding mechanism decrease and the reduction of the activity of digestive enzymes and food absorption [42]. Liu et al. observed the feeding inhibition in *D. magna* exposed to BPA at the concentration of 178 µg/L [22]. The *D. magna* exposed to BPA (178 µg/L) resulted in the expression of acetylcholine esterase activity consequently activity and behavior impairment in the organism. So far, there has been no report on the influence of Pb on food feeding of *Daphnia*, but Pb, at a certain level, would inhibit the feeding activity of the organism because this metal is a neurotoxic chemical impairing the respiratory system and enzyme activities in zooplankton [16, 43]. In contrast, we found the increase of food feeding rate in *D. magna* exposure to BPA+Cd, BPA+Pb+Cd and BPA+Pb (Table 2). The controversial observations between previous studies and ours could be due two potential reasons. Firstly, we used much lower concentrations of metals (5 µg/L) and BPA (50 µg/L) to test with *D. magna* while the concentrations of chemicals for experiments in previous studies were much higher (10 – 100 µg/L of Cd; and 178 µg/L of BPA). The lower concentrations might not induce the inhibitory effect on food feeding activity of *D. magna* in our study. Secondly, the mixtures of BPA and metals (Pb, Cd) may not have an additive or a synergistic effect, but they may result in an antagonistic effect on feeding rate of *D. magna*. 
Lampert [44] proved that Daphnia could grow well at the food concentration from 0.5 mg C/L/day which was approximately 35,000 cells/mL of Scenedesmus [45]. The latter authors noted that one D. magna grew well upon the providing an amount of 700,000 Scenedesmus cells each day as food [45]. In present study, with the feeding rate of 4,870 cells/h (Table 2), one D. magna in the control consumed around 117,000 cells/day which was within the suggested range of food for the organism in a previous investigation [45]. Although the mixtures of metals (Pb, Cd) and BPA could impact on the D. magna (please see the section 3.2 below) hence the chemicals caused the energy cost in the organisms then the organisms would increase their energy demand to deal with toxic chemical challenge [46], the mixtures might not inhibit the feeding activity in a short exposure time (24h). Besides, the toxic chemicals cannot only affect behavior but also the enzyme activities and protein content in D. magna [47]. However, in case of protein degradation in the organisms, they could make a compensation by increasing protein synthesis [46]. Taken together, the exposed D. magna would require more material and energy to compensate the cost, to deal with the damage and to maintain their normal activities. This would lead to the feeding rate increase in the exposed D. magna as observed in this study. Our study reflects the feeding response of D. magna as an adaptive characteristic upon exposures to trace metals and BPA. On the other hand, the influence or the enhancement on feeding rate of D. magna by BPA+Cd was strongest among the exposures. This could be link to the more potent impact of Cd than Pb on D. magna [30, 31] consequently the organisms in BPA+Cd required more energy and material for compensation and maintenance their normal activities, and physiological and biochemical processes in a short time. Hence the D. magna in BPA+Cd exposure fed more food than D. magna in other exposures. The similar food feeding rate of D. magna in the BPA+Pb and BPA+Pb+Cd (Table 2) reflected that Cd slightly impacted the feeding rate under the presence of BPA+Pb. Because the combined effect of BPA and Cd was very strongly on D. magna feeding rate as mentioned above, the Cd seemed to have minor influence on the D. magna feeding upon the presence of Pb. This could be related to the competition between Pb and Cd on the same active site in D. magna as explained with biotic ligand model by Di Toro et al. [48]. The similar free metal ions (Pb²⁺, Cd²⁺) could have the same active binding sites or sites of action in the organism. The competition in binding onto the site lead to the toxic reduction of the mixture metals [48]. Therefore, both Pb and Cd are non-essential and toxic metals to D. magna but their mixture did not increase the feeding rate of the organism (compared to the single metal), hence an antagonistic effect, which needs further investigations to clarify.  

3.3. The effects on the survivorship of Daphnia magna  
After 18 days of the experiment, the survival rate of the control was 100% that strongly met the requirements for a chronic test according to APHA [20]. However, the survival rate of D. magna exposed to the mixtures of these pollutants was significantly lower than that in the control. More details, by the end of the test, the survivorship of D. magna in the BPA+Pb was approximately 37% while the proportion in the BPA+Cd was 10%. Meanwhile, only 50% of total D. magna in the exposure to BPA+Pb+Cd lived at the end of the experiment (Fig 1). We found that in the exposures of BPA+Pb and BPA+Cd the survival rate of D. magna was inhibited more than 60% and 90%, respectively, while the mixture of the three pollutants caused a 50% decline in the survival of the organisms.

The decline in the survivorship of D. magna in the exposures in this study was the evidence demonstrating the adverse effects of these pollutants on the organisms. Being an endocrine-disrupting chemical, BPA is more likely to cause biological effects such as intracellular disruption as well as enzyme activity alteration, rather than lethal effect on the organisms [3, 22, 23, 24]. For instance, Park and Choi showed that although the survival rate did not decrease, the genotoxic effects were found in D. magna exposed to BPA at concentrations from 300 to 30,000 µg/L [23]. Similarly, the mortality of D. magna was not different between the control and exposure to BPA at 10,000 µg/L for 21 days in the study of Jemec et al. [24]. On the other hand, 178 µg/L of BPA could cause the activity decrease of digestive enzymes (trypsin, amylase), antioxidant enzymes (superoxide dismutase, glutathione peroxidase) and the nervous enzyme acetylcholinesterase [22]. Therefore, the BPA at high
concentrations (some hundreds to thousands µg/L) could directly or indirectly alter the normal metabolisms and behavior of *D. magna*.

**Figure 1.** The survival rate of *Daphnia magna* exposed to the mixtures of BPA and trace metals (Pb, Cd) during 18 days of the experiment.

The detrimental effects of Pb on the survivorship of *D. magna* have been showed in the previous study of Luciana et al. [29], in which Pb at the concentration of 90 µg/L could inhibit up to 95% the survival rate of *D. magna* after 15 days of the exposure. Similarly, the survival of *D. magna* was deleteriously impaired in the exposure to 150 µg Pb/L for 21 days [31]. However, survival proportion of *C. magna* was not decreased in exposure to 5 µg Pb/L [31]. Pb has been known as a neurotoxic chemical, thereby exposures to this metal could lead to various negative effects, such as affecting the respiratory system, inhibiting enzyme activities, and consequently the lethal effects on organisms [16, 43]. Regarding the toxicity of Cd, numerous studies have indicated that Cd could adversely affect the survival of *D. magna* even at low concentrations. Bodar et al. [28] found that *D. magna* exposed to Cd at 10 µg/L for 25 days could lead to a serious decline in the survivorship, up to 60%, whereas the report of Perez and Hoang [30] noted that *D. magna* had high mortality, approximately 80%, in the exposure to 1.5 µg/L of Cd.

It is also important to note that the exposed *D. magna* in our study had a higher food feeding rate than usual (Table 2) so that the organisms could ingest more food then have more material and energy during a short time of incubation (24h). However, upon a long period of time continuously exposing to the pollutants, *D. magna* could be severely stressed in biochemical and physiological alterations as reported elsewhere [22]. Then the energy cost due to damage became larger than the energy in the food consumption by the *D. magna*, consequently mortality from the 4th day of incubation (Fig. 1). Although the joint toxicity of BPA, and Pb and Cd has not been reported, the single adverse effects of BPA, Pb and Cd on the survival of organisms could help to explain the decline in the survival rate of exposed *D. magna* in the current study. As mentioned above, the solely BPA (50 µg/L) or Pb (5 µg/L) did not impair *D. magna* survival [23, 31], but we found that the BPA+Pb (mixture) could inhibit up to 63% of the survival rate of *D. magna* in our experiment that was severer than the individual effect of these two pollutants. It seemed that the mixture of BPA and Pb caused a synergistic effect on the survival of *D. magna* in our study. Further studies are suggested to clarify the synergistic effect of BPA and Pb.

In this study, we exposed *D. magna* to BPA+Cd which inhibited up to 90% in the survivorship of *D. magna*. Compared to the previous study, the used concentration of Cd in this study was higher than the concentration of Cd which reduced 80% survival rate of *D. magna* in the study of Perez and Hoang [30]. Therefore, the decline in the survivorship of *D. magna* in the current study could be mainly due to the
toxicity of Cd because BPA at even higher concentration has been demonstrated no effect on the survival of *D. magna* in previous studies [23, 24]. On the other hand, our results showed that the survival rate of *D. magna* exposed to the BPA+Pb+Cd could decline 50% which was less serious than the effect caused by the mixture BPA+Cd, up to 90%. Surprisingly, the occurrence of Pb could reduce the toxicity of the mixture BPA+Cd. This result could be in line with the previous study of Perez and Hoang when the authors found that the occurrence of Zn reduced the toxicity of Cd on the survival of *D. magna* in the exposure lasting 21 days [30]. Similarly, Mahar and Watzin observed the mixture of Zn, Cu, and diazinon causing less toxic than the mixture of the two on the survival of another micro-crustacean species, *C. dubia* [34]. Moreover, the mixture of the three metals including Cd, Cr, and Cu had less toxicity than the mixture of the two metals that have been reported on the copepod *Tisbe holothuriae* [33]. These results could help to explain our observation of the less reduction in the survival rate of *D. magna* exposed to the mixture BPA+Pb+Cd compared to the mixture of BPA+Cd. It was likely that the mixture BPA+Pb+Cd showed an antagonistic effect on the survival of *D. magna*.

3.4. The effects on the maturity age of Daphnia magna

The results on the maturation showed that *D. magna* in the control and in the BPA+Pb matured at a similar age, 4.7±0.4 and 5.4±1.4 days, respectively (p>0.05, Kruskal-Wallis test; Fig. 2). Regarding remaining exposures, we found a significant difference in the maturity age of *D. magna* raised in the BPA+Cd and BPA+Pb+Cd compared to the control (p<0.01, Kruskal-Wallis test). The maturity ages of the organisms in BPA+Cd and BPA+Pb+Cd were 5.5±0.5 and 5.9±1.0 days, respectively (Fig. 2). Thus the mixtures of BPA+Cd and BPA+Pb+Cd caused a delay in the maturation of *D. magna*.

To the best of our knowledge, the joint effects of BPA+Pb, BPA+Cd, and BPA+Pb+Cd, on the maturation of *D. magna* has not been reported so far. Liu et al. found that BPA (178 µg/L) prolonged the day to first brood of *D. magna* [22]. However, Quach et al. reported that Pb (5 µg/L) did not cause the postponement in the maturation of *D. magna* [31]. Therefore, the BPA concentration (50 µg/L) in the mixture of BPA+Pb was not high enough to induce a negative influence on the maturation of the exposed *D. magna* in our study.

![Figure 2](image-url)  
**Figure 2.** Time to maturity of *Daphnia magna* exposed to the mixtures of BPA and trace metals (Pb, Cd) during 18 days of the experiment. The asterisk indicated the significant difference between control and exposures (p<0.01, Kruskal-Wallis test).
Although the impacts of BPA on the maturation of \textit{D. magna} have been not fully understood, this chemical has been known to cause numerous effects related to reproductive activities of the organisms, such as altering expression levels of many genes linking to reproductive processes leading to the inhibition on the reproduction of \textit{D. magna} \cite{25}. Similarly, previous studies have indicated that exposure to Cd could lead to the postponement of the maturity of organisms. For instance, Bodar et al. noted that the Cd at the concentration of 5 µg/L could slightly delay the maturity age of \textit{D. magna} \cite{28}. Moreover, Meng et al. also found that Cd at the same concentration (5 µg/L) in combination with other metals (Pb, Hg, Cr, and Cu) induced a delay in the maturation of \textit{D. magna} in a chronic test \cite{36}. Besides, energy cost in the exposed \textit{D. magna} as mentioned above (section 3.2) would diminish the egg development inside the organism. Summing up, the impacts of a single pollutant (BPA, Pb, Cd) supported our observation on the postponement in the maturity age of \textit{D. magna} exposed to BPA+Cd and BPA+Pb+Cd. Further studies on the biochemical responses of \textit{D. magna} upon exposures to these mixtures to clarify the joint toxicity on the maturation of the organisms.

### 3.5. The effects on the reproduction of \textit{Daphnia magna}

The accumulative neonates of \textit{D. magna} in the control were 1,174 while that in the exposures to the BPA+Pb and BPA+Cd were 1,378 and 301 neonates, respectively. Therefore, the total offspring relative to the control of the mixture BPA+Pb and BPA+Cd were 117% and 26%, respectively (Table 3). On the other hand, the total offspring released from the BPA+Pb+Cd was 923, gaining approximately 79% compared to the control (Table 3). The results on the reproductive performance indicated the influence on reproduction of BPA+Pb+Cd was less than the BPA+Cd but more than the BPA+Pb.

Apparentely, there was a strong decrease of population development of offspring \textit{D. magna} exposed to BPA+Cd and BPA+Pb+Cd (Table 3). However, when combined the number of alive mother \textit{D. magna} (Fig. 1), its maturity ages (Fig. 2) and the total offspring (Table 3) we found no inhibition on the reproductive capacity of mother \textit{D. magna} in the exposures. Because mother \textit{D. magna} matured at around 5 – 6 days old (Fig. 2), the \textit{D. magna} would start to reproduce at the 8th - 9th day of the experiment (data not show). Between the 8th day to the end of experimental period, the proportion of mother \textit{D. magna} in BPA+Pb and BPA+Pb+Cd to control was around 50 – 70% (Fig. 1) and the proportion of their offspring (from the BPA+Pb, BPA+Pb+Cd) to the control was from 79 to 117% (Table 3). Hence the fecundity or brood size of exposed \textit{D. magna} was larger than that of the control. Similarly, the 77 – 90% lower number of mother \textit{D. magna} in BPA+Cd (compared to the control; Fig. 1) produced around 74% lower number of offspring (Table 3). Therefore, we concluded that all the binary and trinary mixtures in present study enhanced the reproductive capacity of \textit{D. magna}, and the enhancement order was BPA+Pb > BPA+Pb+Cd > BPA+Cd.

| Table 3. The accumulative offspring of \textit{D. magna} in the exposures and their proportion to the control |
|---------------------------------------------------------------|
|                  | Control | BPA+Pb | BPA+Cd | BPA+Pb+Cd |
| Accumulative offspring | 1,174   | 1,378  | 301    | 923       |
| Proportion to the control (%) | 100     | 117    | 26     | 79        |

Previous studies showed that BPA did not adversely inhibit the reproduction of the \textit{D. magna}. Jeong et al. found that BPA at the concentration up to 3,000 µg/L did not significantly reduce the number of offspring \textit{D. magna} \cite{25}. Similarly, the reproduction of \textit{D. magna} exposed to 2,000 µg BPA/L was not influenced over the period of 21 days \cite{3}. Jemec et al. \cite{21} and Brenna et al. \cite{49} observed no detrimental effect of BPA at the concentrations of 1,000 – 1,730 µg/L on the brood size and total neonate of \textit{D. magna}. However, the BPA singly at the concentration of 50 µg/L resulted in an increase of total offspring of \textit{D. magna} compared to control (our unpublished data, from a multigenerational experiment).
Therefore, high concentrations of BPA did not enhance the reproduction of the organisms, but it became possible at a lower concentration. This is similar to the influence of another organic compound and also endocrine disrupting compound, phthalate. The phthalate had negative effects on survival (48h-LC50 of 580 µg/L) but the chemical (390 µg/L) strongly stimulated the reproduction in *D. magna*, up to 1.5 times compared to normal [50, 51].

On the other hand, the results of Luciana et al. indicated that the fecundity of *D. magna* was not reduced in the exposure to Pb at the concentration up to 30 µg/L which was much higher than the used concentration of Pb in the current experiment (5 µg/L) [29]. In addition, Quach et al. found that the reproduction of *D. magna* was not inhibited, even slightly stimulated in the exposure to Pb at the same concentration as used in this study (5 µg/L) [31]. In the current study, the used concentrations of BPA and Pb were within the range or even lower than the no-effect concentrations reported in previous studies. Bodar et al. and Perez and Hoang found that the number of offspring *D. magna* in the exposure to Cd (1.5 – 10 µg/L) for 21 days significantly reduce [28, 30].

Therefore, beside the dead organism, the alive *D. magna* exposed to mixtures of BPA and the metals could suffer from the joint effects on reproduction. As mentioned above, both BPA and Pb separately enhanced the reproduction of *D. magna* so their mixture would strongly stimulate the accumulative offspring of *D. magna*. The metal Cd prevented the reproduction of *D. magna* hence the organisms exposed to BPA+Pb+Cd would produce less offspring than those exposed to BPA+Pb did. Then the mother *D. magna* treated with BPA+Cd would produce less offspring as noted in the Table 3. Moreover, the results from the food feeding rate experiment could be an evidence for the energy cost and more material demand for compensation in the exposed *D. magna*. It is reasonable that the more spending energy against the impacts of BPA and metals, and the reproductive capacity maintenance and/or enhancement in the exposed *D. magna* would lead to a higher food demand consequently higher food feeding rates in the exposed organisms as in the Table 2.

The total offspring reduction in the exposures would not be a good outcome because this evidenced for the severe impacts of the mixtures of BPA and the metals. The environmental relevant concentrations of BPA, Pb and Cd not only impaired the survival of mother *D. magna* but also inhibited the population size of offspring *D. magna*. Therefore, in the nature, the influences of mixed pollutants over a longer time (e.g. more than three weeks) and across multi-generations of the *D. magna* would lead to an extinction of the micro-crustacean population, consequently imbalanced aquatic ecosystems. Investigations on the impacts of these pollutants on zooplankton in nature are suggested.

4. Conclusions
Our results revealed that exposures to the mixtures of BPA and the two metals (Cd and Pb) could result in numerous effects on the feeding activity and life history traits of the freshwater micro-crustacean, *D. magna*. In terms of acute exposure, we found that all the mixtures of these chemicals (BPA+Pb, BPA+Cd, and BPA+Pb+Cd) strongly stimulated the feeding activity of the organisms. On the other hand, the survival rate of *D. magna* in the chronic exposure to these mixtures was significantly declined. Besides, although the mixture of BPA and Pb did not adversely affect the maturation and reproductive performance of *D. magna*, exposures to the BPA+Cd and BPA+Pb+Cd could lead to an impairment in the reproduction and a delay in the maturity age of the organisms. Regarding joint toxicity, we found the synergistic effects of BPA+Pb on the survival and reproduction of *D. magna*, but there was an antagonistic effect of Pb+Cd on the survival of the organisms (stronger impacts of BPA+Cd than BPA+Pb+Cd). Our findings indicated that the combinations of the two metals Cd and Pb at the permissible levels (according to the technical regulation for surface water quality of Vietnam; QCVN) and the plastic additive BPA (which has not been included in QCVN) could cause detrimental effects on the organisms. Therefore, further investigations should be conducted to adjust the safety guidelines related to these pollutants for environmental and ecological protection. Our results not only enrich the
knowledge on the combined toxicity of BPA and the two non-essential metals (Pb and Cd) but also could be valuable for monitoring and risk assessments of these pollutants in aquatic environment.

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