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Single and combined effects of Di-2-ethylhexyl phthalate and bisphenol A on life traits of the tropical micro-crustacean *Ceriodaphnia cornuta*

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Abstract:

Plastics, plastic additives, and their emission have attracted significant attention and concern both socially and scientifically. Di-2-ethylhexyl phthalate (DEHP) and bisphenol A (BPA) are two of the many plastic additives widely found in aquatic environments, which can have severe impacts on aquatic animals like micro-crustaceans. Therefore, this study assessed the chronic effects of DEHP and BPA, both individually and jointly, at environmental concentrations (e.g. 50 and 500 µg/l) on the survival rate, reproduction, and growth of the tropical micro-crustacean *Ceriodaphnia cornuta*. We found that each of the two plastic additives, and a mixture of the two, had some influence on the survivorship of *C. cornuta*. While DEHP marginally enhanced the reproduction of the animals, BPA strongly inhibited it. Additionally, the mixture of DEHP and BPA caused a synergistic effect on reproduction but an antagonistic effect on the growth of *C. cornuta*. Both DEHP and BPA induced a significantly longer body of *C. cornuta* when exposed to these plastic additives. Our results showed that the tropical micro-crustacean *C. cornuta* is more sensitive to DEHP and BPA than the temperate micro-crustacean *D. magna* in relation to body length development and reproductive characteristics. Our findings enrich the knowledge of DEHP and BPA toxicity to tropical micro-crustaceans. Besides, our results are also of significant value to freshwater monitoring and environmental risk assessments of plastic additives.

Keywords: **energy cost, plastic additives, synergistic effects, tropical micro-crustacean.**

Classification number: **5.1**

Introduction

The global production of plastic has continuously increased over the past few decades as production reached nearly 360 million tons in 2018 [1]. However, only less than 5% of plastic materials have been recovered [2]. Consequently, the accumulation of plastic in the environment, especially in water bodies, has rapidly increased and become a critical concern for the environment, ecosystems, and human health due to the persistence and non-biodegradability of plastic waste [3, 4]. Further, plastics can contain various harmful chemicals like plastic additives (e.g. phthalate, bisphenol A, etc.) that are added to plastic polymers to provide them with specific characteristics like making them harder, more flexible, and/or durable [5, 6]. However, in the environment, these chemicals enter water bodies through pathways like discharge from industrial manufacturing or even by leaching out of the plastic materials themselves during their use and disposal, which can be magnified under natural conditions such as high temperature and UV radiation [7, 8]. Indeed, the potential release of hazardous additives from plastic materials has been demonstrated in several studies performed under laboratory conditions [9-11]. For instance, a BPA concentration of up to 8.3 µg/l was found in drinking water stored in polycarbonate bottles [9]. Similarly, common phthalate derivatives such as DEHP, dibutyl phthalate (DBP), and diisobutyl phthalate were detected in liquid extracts from polymer-coated materials in a study by Bradley, et al. (2007) [10]. Phthalates and BPA are widely used in the manufacture of plastic and frequently

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detected in the aquatic environment. For example, phthalates are mainly used in polyvinyl chloride (PVC) manufacturing while BPA is commonly used as a monomer, an antioxidant, and/or a plasticizer for epoxy resins, polycarbonate (PC), PVC, polypropylene (PP), and polyethylene (PE) plastics [7]. The global production of these additives has continuously increased due to an increase in plastic consumption, which has reached approximately 8 million tons per year [12, 13].

Due to their wide application, huge demand, and mismanaged disposal, plastic products containing phthalates and BPA have led to an increase in concentration of these additives in the environment [12-15]. The concentration of BPA can be higher than 90 µg/l in surface water and up to 370 µg/l in wastewater [12, 16]. Besides, DEHP, the most commonly detected phthalate in aquatic environments, can reach 370 µg/l in surface water [13]. Plastic additives such as phthalates and BPA are known as endocrine-disrupting compounds (EDCs) that can cause intracellular disruption and interfere with the functions of hormones in the endocrine systems of living organisms [15, 17, 18]. Previous studies have reported negative impacts of these additives on aquatic organisms such as phytoplankton, zooplankton, and fish [12-15, 19, 20]. For instance, DBP can alter lipid content causing an inhibition of the growth of the green alga *Chlorella vulgaris* [21]. The work of Wang, et al. (2018) [13] indicated that DEHP can strongly influence biochemical and physiological activities of the micro-crustacean *Daphnia magna* by enzymatic inhibition, increasing lipid peroxidation levels, and modulating the transcription of enzyme levels. Moreover, phthalates can inhibit the absorption and catabolism of fatty acids and cause detrimental effects on the development, reproduction, and lifespan of *D. manga* [5]. Similarly, the detrimental effects of BPA on the enzymatic activity, lipid peroxidation level, and reproduction of *D. magna* has been demonstrated in a study by Jemec, et al. (2012) [22]. Further, exposure to BPA can cause DNA damage resulting in a genotoxic effect on *D. magna* [23]. The acute toxicity of BPA has also been reported on the tropical micro-crustaceans *Ceriodaphnia silvestrii* and *Daphnia similis* with a 48 h-EC_{50} value of 14.44 and 12.05 mg/l, respectively [14]. Moreover, numerous studies have shown that phthalates and BPA have inhibitory effects on the reproduction, development, and enzymatic activity of various fish species, as well as cause their malformation [20, 24-26]. Therefore, the presence of these additives is considered a potential risk of biological disorder in animals and ecological imbalance in aquatic environments.

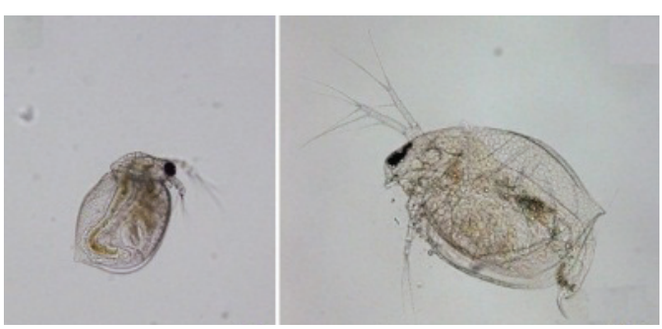
In aquatic ecosystems, zooplankton (e.g. *Daphnia*, *Ceriodaphnia*) play an important role as they are centrally positioned in the food chain and are among the most vulnerable organisms to pollution [27]. These organisms are commonly used in toxicological assessments due to their wide distribution in aquatic ecosystems, high sensitivities to toxins, and ease of culture under laboratory conditions [28-31]. Although previous studies have shown the negative impacts of phthalates and BPA on various aquatic organisms, the chronic effects of these chemicals on zooplankton from tropical regions have not been fully understood. Therefore, the aim of this study is to assess the chronic impacts of DEHP and BPA on the survival, reproduction, and growth of the tropical micro-crustacean *Ceriodaphnia cornuta* isolated from Vietnam.

Materials and methods

The test organism and chemicals for the experiment

The tropical micro-crustacean, *C. cornuta* (Fig. 1), was isolated from the Mekong river in Vietnam and was maintained for over one year under laboratory conditions at a temperature of 25±1°C, light intensity of 600 lux, and photoperiod of 12 h light: 12 h dark [30, 32]. The organism was raised in an artificial medium called M4/4 [30] and fed a mixture of the green alga *Nannochloropsis* sp. and YTC, a rich nutrient mixture [33]. The alga was cultured in Z8 medium [34] under the laboratory conditions mentioned above.

The plastic additives DEHP and BPA, from Aldrich Sigma, were dissolved in acetone (Merck) at concentrations of 1000 and 5000 mg/l, respectively, and used as the mother solutions for the experiments. The mother solutions were stored at a temperature of 4°C prior to the experiment.



(A) (B)

Fig. 1. (A) The neonate and (B) the adult *Ceriodaphnia cornuta*. Scale bars = 200 µm.

Experimental setup

Prior to the experiments, more than 30 healthy mother *C. cornuta* were randomly selected and incubated in 50 ml beakers containing 30 ml M4/4 medium (3 individuals/ beaker). The neonates (less than 24 h old) from these beakers were used for chronic experiments. The chronic experiments were conducted according to APHA (2012) with minor modifications [32]. Briefly, the neonates of *C. cornuta* (less than 24 h old) were randomly collected and exposed to DEHP and BPA at concentrations of 50 and 500 µg/l. Another test was conducted in which the animals were exposed to a mixture of DEHP and BPA at a concentration of 50 µg/l (for each chemical). The control was conducted in parallel with the exposures by culturing the organisms in the M4/4 medium without the addition of plastic additives (Table 1). The concentrations of DEHP and BPA in our study are within the range of the chemical concentrations found in the environment [12, 13, 16].

Table 1. Summary of the chronic exposures of *Ceriodaphnia cornuta* to DEHP and BPA.

No.	Abbreviations of the exposures	Concentrations of DEHP (µg/l)	Concentrations of BPA (µg/l)
1	Control	0	0
2	D50	50	0
3	D500	500	0
4	B50	0	50
5	B500	0	500
6	Mix	50	50

For each treatment, the organism was individually incubated in a 15 ml glass tube containing 10 ml M4/4 medium at the test concentration of chemicals (one organism/tube). There were 10 replicates (n=10) in each treatment. The experiments were performed under the laboratory conditions as mentioned above and lasted for 10 d. The organisms were fed daily with a mixture of green alga *Nannochloropsis* sp. and YTC [33]. The medium in each incubation was totally renewed three times per week. During the experimental time, the life-history traits including survivorship and reproduction of *C. cornuta* were recorded daily over a period of 10 d. By the end of the test, the body length of the living organisms in each treatment was measured by using a microscope (Olympus BX 51) coupled with a digital camera (DP71) [31].

Data treatment

Sigma Plot Version 12.0 was used for data analyses. The ANOVA test was applied to calculate the statistically significant difference in the body length of *C. cornuta* between the control and exposures. A gap of more than 20% in the survival proportion of *C. cornuta* in the treatments was considered as a significant difference [32].

Results and discussion

Effects of DEHP and BPA on the survivorship of Ceriodaphnia cornuta

By the end of the test, more than 90% of total organisms in the control treatments were still alive (Fig. 2), which was in line with the requirement for chronic experiments according to APHA (2012) [32]. During the exposure to DEHP, none of *C. cornuta* died until the end of the incubation, while the survival rate of organisms exposed to BPA at the concentration of 50 µg/l (B50) and 500 µg/l (B500) was 80 and 100%, respectively (Fig. 2A, B). Similarly, 80% of the total *C. cornuta* incubated in a mixture of DEHP and BPA (mix) were still alive at the end of the experiment (Fig. 2C). In this study, the difference in the survivorship of the organisms in all the DEHP and BPA exposures compared to the control were not statistically significant according to APHA (2012) [32]. Hence, the exposures to the individual and mixture of DEHP and BPA at the test concentrations during the 10-d period did not negatively influence the survival of the tropical micro-crustacean *C. cornuta*.

Our results were in line with previous studies reporting that DEHP at concentrations from 158-500 µg/l did not impact the survival rate of *D. magna* during 21 d of incubation [35, 36]. Spadoto, et al. (2017) [14] found that no observed effect concentration of BPA on the tropical micro-crustacean *C. silvestrii* was 1380 µg/l upon 8 d of exposure. Additionally, the authors also showed that the hazardous concentration for 50% of *C. silvestrii* was 493 µg BPA/l, which supports our observation of the survival rate of *C. cornuta* treated with BPA (up to 500 µg/l) in the current study. We expect that the toxicity of the mix (50 µg DEHP/l and 50 µg BPA/l) on *C. cornuta* survival would not be stronger than the highest single chemical treatment (either D500 or B500) and this is confirmed in the current study (Fig. 2). Most likely our study confirms that the EDCs DEHP and BPA at the test concentrations had no significant effects on the survival of a single or parent generation of tropical micro-crustaceans [14].

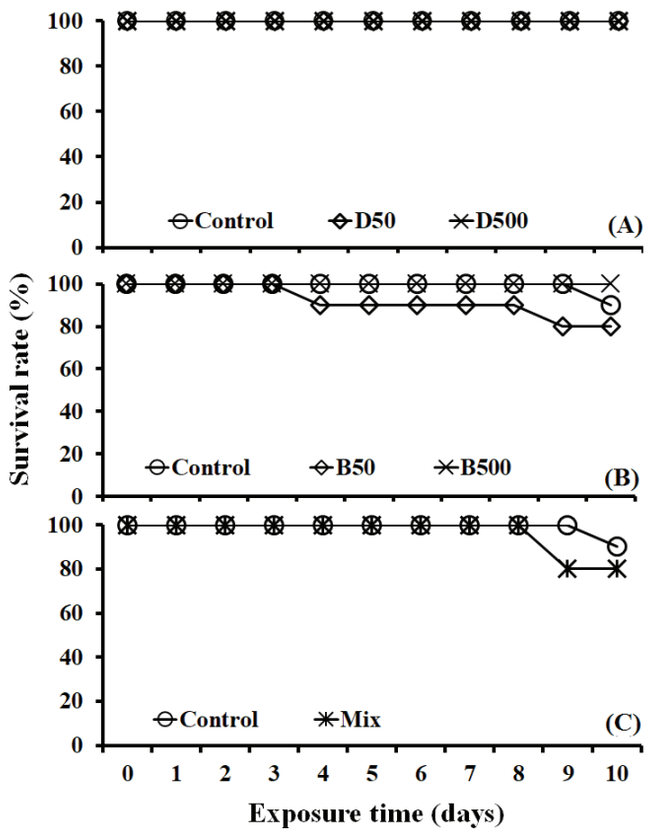


Fig. 2. The survival rate of *Ceriodaphnia cornuta* exposed to (A) DEHP, (B) BPA, and (C) a mixture of DEHP and BPA. D50 and D500 correspond to the medium containing 50 and 500 µg/l of DEHP, respectively, while B50 and B500 correspond to the medium containing 50 and 500 µg/l of BPA, respectively. Mix denotes the medium containing 50 µg DEHP/l and 50 µg BPA/l (Table 1).

Effects of DEHP and BPA on the reproduction of Ceriodaphnia cornuta

After 10 d, the total offspring of *C. cornuta* in the control, D50, and D500 trials were 75, 80, and 86 neonates, respectively. Therefore, the total neonates of D50 and D500 (or reproduction relative) to the control were 107 and 115% (Fig. 3A). On the contrary, in the BPA treatments, the reproduction relative to the control of B50 and B500 were 21 and 22%, respectively (Fig. 3B). The reproductive performance of *C. cornuta* incubated in the mixture of DEHP and BPA was strongly inhibited and gained only 5% compared to the control (Fig. 3C).

Knowles, et al. (1987) [35] and Le, et al. (2019) [36] found that DEHP at concentrations in the range of 50-158 µg/l did not reduce nor enhance the reproduction of *D. magna*. However, a higher DEHP concentration of 390 µg/l resulted in a 1.5-times higher reproduction of *D. magna* compared to the control [15]. The reproduction relative to the control in *C. cornuta* (115%, Fig. 3A) at a DEHP concentration of 500 µg/l was similar to that of *D. magna* (112%) in a previous investigation [36]. This similarity is likely due to both species being micro-crustaceans.

However, the reproduction of *C. cornuta* in the present study was strongly impacted by BPA at both concentrations tested (50 and 500 µg/l, Fig. 3B). This is contrary to the results from a study on another tropical micro-crustacean species *C. silvestrii*, where its fecundity was observed to not be significantly reduced after exposure to 1380 µg BPA/l [14]. Jemec, et al. (2012) [22] found that the no observed effect concentration of BPA on the brood number and total offspring of *D. magna* were 860 and 1730 µg/l, respectively. The brood size of *D. magna* was not impacted by BPA concentrations up to 1380 or even 6900 µg/l [14, 22]. Therefore, it would seem that the reproductive trait of *C. cornuta* from the Mekong river in Vietnam is more sensitive to BPA than that of *C. silvestrii* and *D. magna.* Although DEHP and BPA are both EDCs, they can have opposite effects on the reproduction of micro-crustaceans from the same species, for example, *C. cornuta* in this case. Another study found that BPA at low concentration (e.g. 3 µg/l) could cause DNA damage and significant changes in antioxidant enzyme activities (e.g. catalase) in *D. magna* [23]. The activity of the biotransformation enzyme also significantly increased upon a chronic BPA treatment [22]. Hence the BPA exposures could lead to an energy cost in micro-crustaceans (e.g. *D. magna*). The energy cost, over a chronic treatment, could diminish the reproductive capacity in the exposed animals, which may help to explain the strong reduction in the total neonates of *C. cornuta* in our study. The biochemical responses of *C. cornuta* upon incubation in BPA and DEHP are suggested for further studies.

The relative reproduction of *C. cornuta* in D50, B50, and the mixture to the control (Fig. 3) were 107, 21, and 5%, respectively, which revealed that the mixture of BPA and DEHP resulted in a synergistic effect on the reproduction of the exposed animals. Similarly, Baralic, et al. (2020) [37] reported that a mixture of phthalates (DEHP, DBP) and BPA induced more pronounced effect on a cellular level in mammals than the chemicals individually (DEHP, DBP, BPA). However, to the best of our knowledge, there has been no report on the combined effects of DEHP and BPA on aquatic animals. Apparently, both DEHP and BPA

can strongly alter the antioxidant and biotransformation enzyme activities in micro-crustaceans [22, 23, 35] leading to an energy cost over chronic exposures. This would then imbalance the energy distribution that the animals use to maintain their survival, conduct normal activities such as swimming and feeding, and for their growth and reproduction. Specifically, the impairment of the reproduction of *C. cornuta* by BPA was evidenced in this study (Fig. 3B). Hence, an increase in reproductive function impairment would occur upon exposure to BPA and DEHP, however, it is not clear if DEHP impacts other functions or if it just simply causes further energy cost in the animal.

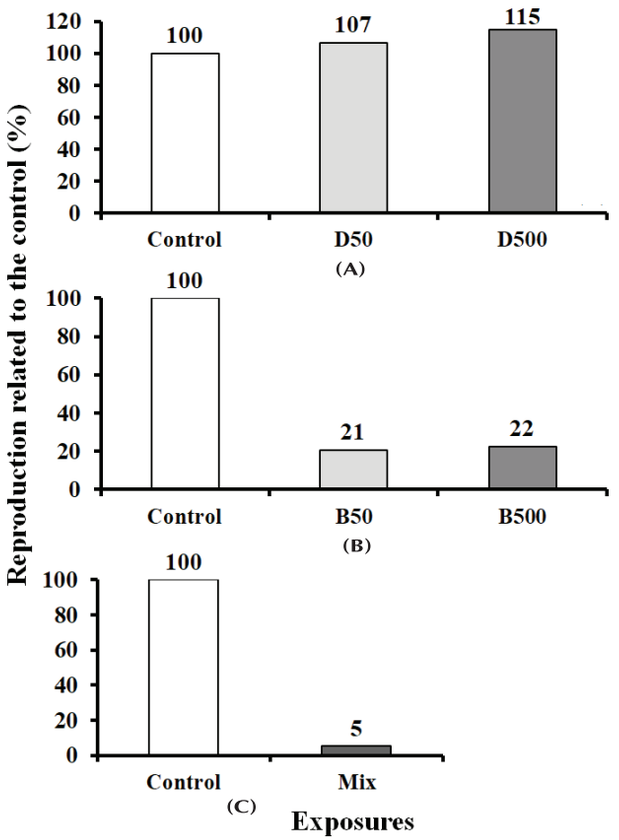


Fig. 3. Total neonates of *Ceriodaphnia cornuta* exposed to (A) DEHP, (B) BPA, and (C) a mixture of DEHP and BPA relative to the control. Abbreviations as in Fig. 2.

Effects of DEHP and BPA on the growth of Ceriodaphnia cornuta

After 10 d, the mean body length of *C. cornuta* in the control was 0.458(±0.024) mm. In the DEHP and BPA treatments, the body lengths of the animals were significantly longer than that of the control. Briefly, the mean body length in D50, D500, B50, and B500 was 0.522(±0.043), 0.532(±0.035), 0.520(±0.045), and 0.532(±0.035) mm, respectively (Fig. 4A, B). Interestingly, the body length of *C. cornuta* in the DEHP and BPA mixture was 0.459(±0.044) mm, which was similar to that of the control (Fig. 4C).

Interestingly, the present results from the DEHP treatments are contrary to previous studies of the micro-crustacean *D. magna* [15, 36] in which DEHP at a concentration of 50 µg/l neither enhanced nor inhibited the body length of *D. magna* at concentrations between 390-500 µg/l. Park & Choi (2009) [23] observed similar body fresh weights of *D. magna* between the control and BPA exposure experiment at a concentration of 30 µg/l. Similarly, Jemec, et al. (2012) [22] did not find any statistically significant change to the body length of *D. magna* exposed to 6900 µg/l BPA. However, in our study, *C. cornuta* growth was stimulated and its body prolonged when exposed to a BPA concentration of 50 µg/l. Therefore, we conclude that the tropical micro-crustacean *C. cornuta* has a much different response to DEHP and is more sensitive to BPA than the temperate micro-crustacean *D. magna* in relation to the body length of the animals.

Differing from the individual exposures to either DEHP or BPA, the mixture of DEHP and BPA in our study did not significantly change the body length of the *C. cornuta*. Hence, these results from the mixture demonstrated antagonistic effects on the body length of the micro-crustacean. It is not completely understood how the mixture of DEHP and BPA prevented body length prolongation compared with the exposure to the individual chemical. However, we can outline some potential causes: 1) a significant increase of energy cost; 2) a potentially competitive binding mechanism; and 3) both energy cost and binding mechanism competition in the animals. As mentioned above, both DEHP and BPA could induce an energy cost and the combined cost of these plastic additives would strongly reduce the energy for not only reproduction but also growth. Considering this, the body length development would be slower than that when exposed to a single plastic additive (DEHP or BPA). Undoubtedly, DEHP or BPA would bind to specific ligand(s) in the micro-crustacean before inducing its effects. For example, the competitive binding mechanism of the metals Cd and Ni to the biotic ligand in *D. magna* was reported by Perez & Hoang (2018) [38] in which the metal Ni (less toxic to *D. magna*) would compete with Cd (more toxic to *D. magna*) to bind to the same biotic ligand. This competition

led to a reduction of Cd toxicity to *D. magna*. From this study, one could infer that DEHP and BPA bind to the same biotic ligand in the micro-crustacean *C. cornuta*; one that is closely linked to body length development. However, the latter hypothesis needs further investigations to clarify.

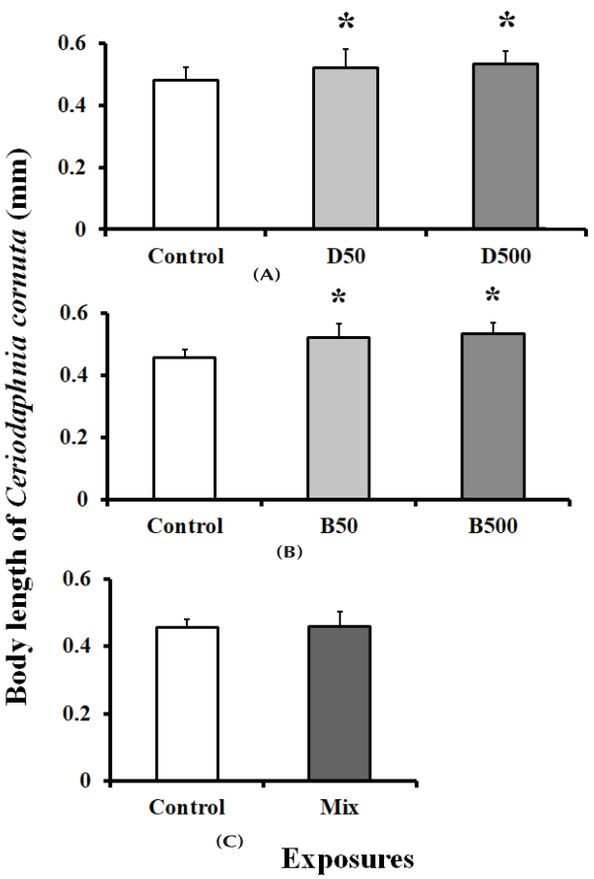


Fig. 4. Body length of *Ceriodaphnia cornuta* exposed to (A) DEHP, (B) BPA, and (C) a mixture of DEHP and BPA. The asterisk indicates a significant difference between the control and exposures ($p<0.05$) by ANOVA followed by Tukey's test. Abbreviations are the same as in Fig. 2.

Conclusions and recommendation

The two plastic additives DEHP and BPA, along with their mixture, did not strongly affect the survival rate of the tropical micro-crustacean *C. cornuta*. While DEHP at the test concentrations only slightly enhanced the reproduction of *C. cornuta*, it significantly boosted their growth. Differently, BPA exposure resulted in faster body length development but inhibited the reproduction of the animals. The mixture of DEHP and BPA had a synergistic effect on the reproductive capacity and an antagonistic effect on the body length development of *C. cornuta*. Energy cost and biotic ligand competition could be the mechanisms behind the observed impairments in the animals exposed to DEHP and BPA. We found that the tropical micro-crustacean *C. cornuta* is more sensitive to DEHP and BPA than the temperate micro-crustacean *D. magna* in relation to body length development and reproductive characteristics. Further investigations of the biochemical responses of *C. cornuta* after exposure to DEHP and BPA are suggested. Our results enrich the knowledge of DEHP and BPA toxicity in tropical micro-crustaceans and are valuable for freshwater monitoring and environmental risk assessments of plastic additives.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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