TRANSGENERATIONAL EFFECT OF DI (2-ETHYLHEXYL) PHTHALATE (DEHP) EXPOSURE ON THE GROWTH AND REPRODUCTION OF *MOINA MACROCOPA* (CRUSTACEA: CLADOCERA)

CHAIKRITSADAKARN, A.[1] – WITTHAYAWIRASAK, B.[1] – MUENPO, C.[2] – DELAUNE, R. D.[3] – MUENHOR, D.[1,4,5*]

[1]*Faculty of Environmental Management, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand*

[2]*Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand*

[3]*Department of Oceanography and Coastal Sciences, Louisiana State University, Baton Rouge, LA 70803, USA*

[4]*Center of Excellence on Hazardous Substance Management (HSM), Bangkok 10330, Thailand*

[5]*Health Impact Assessment Research Center, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand*

**Corresponding author*
e-mail: dudsadee.m@psu.ac.th, mdudsadee@gmail.com

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Abstract. Di-(2-ethylhexyl) phthalate (DEHP) is extensively used in plastic, which results in DEHP to appear in numerous environmental media worldwide. There is a lack of sufficient studies on the long-term transgenerational effect of DEHP exposure on the growth and reproduction of *M. macrocopa.* While non-fatal effects of DEHP exposure in *M. macrocopa* have been reported earlier, this study elucidates that DEHP exposure to parental water fleas had no adverse effects on offspring development or capacity for reproduction. The exposure for the parental generation through F2 progeny did not result in a concentration-dependent growing deleterious effect in subsequent generations, even with long-term exposure. These findings imply that DEHP exposure can cause oxidative stress, and that water flea (*M. macrocopa*) growth and reproduction were not significantly different from blank control and solvent control. The results of the present study aid in the understanding of DEHP risk assessment in aquatic systems. Moreover, the findings can be applied as baseline data to carry out future research on ecotoxicological risk assessment of DEHP.

Keywords: *di (2-ethylhexyl) phthalate (DEHP), Moina macrocopa, sublethal toxicity, transgenerational effect, ecotoxicological risk assessment*

Introduction

In polyvinylchloride (PVC) polymers, phthalates are employed as a plasticizer. The most hazardous phthalate to living organisms is di (2-ethylhexyl) phthalate (DEHP) (Heudorf et al., 2007; NTP-CERHR, 2006). DEHP can leach, migrate, or evaporate into numerous environmental compartments as a result of the widespread usage of DEHP and phthalate plasticizers that are not chemically attached to PVC (Wittassek et al., 2011). DEHP can be found in the air, indoor dust, floor, and road dust, among other environments (Gevao et al., 2013; Muenhor et al., 2018). In various regions of Thailand, aquatic systems have been documented to be contaminated with DEHP of 8.64 μg/L for

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water samples from the source area, deltas of major rivers discharging into the Gulf of Thailand (Sirivithayapakorn and Limtrakul, 2007), and 1.28-5.28 μg/L in surface water U-Tapao canal, Songkhla, Thailand (Kingsley and Witthayawirasak, 2020).

Numerous studies have revealed that DEHP, a persistent organic pollutant in aquatic habitats, is hazardous to many species (Alfardan et al., 2018; Chen et al., 2015; Dombret et al., 2017; Grande et al., 2006; Hirai et al., 2015; Hopf et al., 2014; Jeddi et al., 2016; Li et al., 2015; Lyche, 2009; Uren-Webster et al., 2010; Zanotelli et al., 2010). DEHP was categorized as a developing endocrine disruptor and epigenetic toxicant as a result of its negative impacts on growth and reproduction (ATSDR, 2002). Conversely, DEHP can increase the antioxidant enzyme activity in aquatic creatures like fish (Huang et al., 2015; Kaptaner et al., 2020; Mankidy et al., 2013; Qu et al., 2015; Yuan et al., 2017), zooplankton *Daphnia magna* (Wang et al., 2018), and pearl oyster (Xiang et al., 2017). Studies on DEHP sublethal concentration and chronic exposure in aquatic creatures are scarce compared to studies on DEHP exposure in vertebrates. Moreover, only few studies expand on the transgenerational effect of DEHP exposure on the growth and reproduction of zooplankton *Moina macrocopa* (Crustacea: Cladocera).

The freshwater macroinvertebrate water flea, or *Moina macrocopa* (*M. macrocopa*), is a native species in Thailand. Aquatic food webs in ecosystems on the epicontinent make considerable use of populations of this species (Iannacone and Alvariño, 2000). *M. macrocopa* is susceptible to contaminants, it makes a useful animal model for ecotoxicological research (Park and Choi, 2008) and has a brief life cycle of about 7 days under optimal circumstances (Oh and Choi, 2012). DEHP represents high risk of endocrine disruption in aquatic organisms and ecotoxicological risk assessment of DEHP has been reported by several international authorities (EU, 2001, 2019; U.S. EPA, 1999; WHO, 1992). Consequently, this study details the sublethal consequences of DEHP from ongoing exposure to *M. macrocopa*. Furthermore, in order to better understand how *M. macrocopa* is affected by DEHP exposure across three generations, the toxic endpoints of growth and reproduction were examined.

Materials and methods

DEHP preparation

DEHP (>98% purity) was purchased from Sigma-Aldrich, St. Louis, MO, USA. By dissolving in 0.1 v/v dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) of DEHP, the stock solution was created.

Culture condition

In each test, a single *M. macrocopa* clone was utilized. The *M. macrocopa* breeding stock was maintained as a pure parthenogenetic culture in a 250 mL beaker of culture medium which contained freshwater (pH 7-8; total hardness above 140 mg $CaCO_3$/L; dissolved oxygen concentration > 3 mg/L). Pathum Thani Inland Aquaculture Research and Development Center, Department of Fisheries provided the formula for the culture media. *M. macrocopa*'s culture media contains 0.4 g/L of fertilizer in the form of urea (46-0-0), 0.4 g/L ammonium phosphate fertilizer (16-20-0), 0.6 g/L $CaOH_2$, and 1.2 mL monosodium glutamate effluent. Fresh *Chlorella* sp. was used to feed *M. macrocopa*. The food density of *Chlorella* sp. ranged between 1.2×10^5 and 1.4×10^5 cells/mL. The photoperiod was 12L:12D, and there was an average light intensity of 3.6 MJ/m^2 each

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day. The temperature was 28.5°C. *M. macrocopa* was exposed to each treatment for approximately 24 h in healthy newborns. The exposure containers were 250 mL beakers containing 50 mL of solution in each, and they were used in the same conditions as above.

Sublethal toxicity test (chronic exposure)

From the first post-hatch day (24 h after hatching) until postnatal day 8, *M. macrocopa* were exposed to different concentrations of DEHP. Four different DEHP doses were applied to the test organisms in a serial dilution ratio. In this study, 0.1%v/v DMSO was an appropriated diluent, 0.1%v/v DMSO was considered to be safe for almost all cells (Chen and Thibeault, 2013). The maximum value for *M. macrocopa* was 4410 g/L according to the results of the 24 h LC_{50} of DEHP exposure in aquatic living organisms (Waruthamaphan and Witthayawirasak, 2013); DEHP pollution of the environment has been observed, ranging from undetectable concentrations to 97.8 g/L DEHP in freshwater (Fromme et al., 2002; Long et al., 1998; Sirivithayapakorn and Limtrakul, 2007; Tan, 1995; Vitali et al., 1997; Yuan et al., 2002), 1.74–182 μg/L DEHP in municipal wastewater (Boonyatumanond et al., 2002; Staples et al., 1997). As a result, *M. macrocopa* was exposed to four sublethal concentrations of DEHP: 0 as a blank control, 0.1% v/v DMSO as a solvent control, 1/1000, 1/100, 1/10 folds, and 1 fold of 1000 g/L. The dosage group was tested over three replicates with three *M. macrocopa* per treatment in each replication (0, 1, 10, 100, and 1000 g/L DEHP). In each 250 mL glass beaker with a 50 mL test solution, one neonate (24 h after hatching) was put to the test. The environmental conditions were comparable to the culture conditions in which *M. macrocopa* was fed with *Chlorella* sp. The food density of *Chlorella* sp. ranged between 1.2×10^5 and 1.4×10^5 cells/mL. Tests were performed in a manner similar to that of culture. Following exposure, *M. macrocopa*'s growth and reproduction were monitored daily to see whether long-term exposure to DEHP had any negative consequences (around 8 days after exposure or until the end of lifespan).

(1) Effect of DEHP on M. macrocopa growth

At least three repetitions of each dosage group (0, 1, 10, 100, and 1000 g/L DEHP) were evaluated. Three *M. macrocopa* were used in each replication for each treatment. In each 250 mL glass beaker with a 50 mL test solution, one neonate (24 h after hatching) was put to the test solution and exposure condition was similar to the culture condition. The body length at the mature growth stage which was two days following exposure, was used to measure *M. macrocopa* growth. Then, using an optical microscope with a 5x objective lens (Motic, China) and eyepiece camera TC3100 (Xenon, China), photos were taken 48 h after exposure to measure the adult *M. macrocopa*'s body length. ToupView 3.7, an image analysis program, was used to examine the recorded photos and calculate the body length from head to tail.

(2) Effect of DEHP on M. macrocopa reproduction

At least three repetitions of each dosage group (0, 1, 10, 100, and 1000 g/L DEHP) were evaluated. Three *M. macrocopa* were used in each replication for each treatment. In each 250 mL glass beaker with a 50 mL test solution, one neonate (24 h after hatching) was put to the test solution and exposure condition was similar to the culture condition. Additionally, *M. macrocopa* was exposed to DEHP during its entire life. The total number of newborns produced by each female, measured in offspring per female,

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was among the reproductive features that were observed. After determining the size of the brood, the newborns were separated from the mother. It was done again and again till the females passed away. After Boxplot had reviewed the data, outliers or incorrectly detected water fleas had been eliminated.

(3) Transgenerational effect

At least three repetitions of each dosage group (0, 1, 10, 100, and 1000 g/L DEHP) were evaluated. Three *M. macrocopa* were used in each replication for each treatment. One neonate was examined per 250 mL glass beaker that held a 50 mL test solution (24 h after hatching). The exposure condition was similar to the culture condition. Furthermore, *M. macrocopa* was exposed to DEHP for its whole lifespan. *Figure 1* described the experimental design of DEHP exposure for the multigenerational test of reproduction and growth in *M. macrocopa* under above conditions. The P0 ancestors to the second filial generation (P0, F1, F2) *M. macrocopa* were treated with DEHP solution at a single dose of 0, 1, 10, 100, and 1000 µg/L per day. Offspring (F1) were exposed to DEHP, followed by the F2 offspring also exposed to DEHP to produce a third-generation offspring (F3). Then, F3 offspring were removed from initial DEHP exposure to the normal medium without DEHP. All reproductive characteristics of F3 *M. macrocopa* were counted.

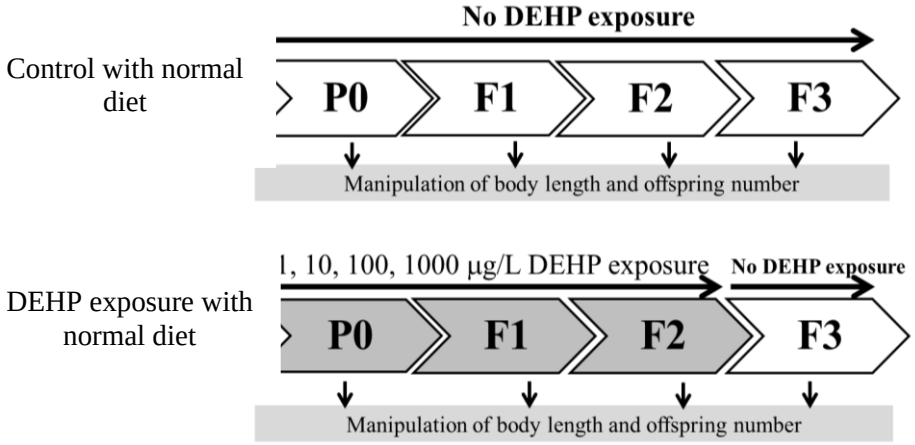

Figure 1. *Experimental design of DEHP exposure for the multigenerational test of reproduction and growth in water flea (M. macrocopa). Maternal water fleas (P0) were exposed to different concentrations of DEHP, which were 1, 10, 100, and 1000 µg/L DEHP, respectively. Subsequently, the P0 water fleas at day 1 adulthood were transferred to the same concentration of DEHP-contained medium with normal diets for 8 days so that P0 could not produce offspring. Then, allowed P0's offspring (or F1) to reach adulthood for 1 day and then counted offspring number. Moreover, the offspring of P0 (F1) were treated with the same concentration of DEHP with the maternal exposed condition. For the next generation, F1 water fleas were placed in the same DEHP contained medium with normal diets and the procedures were performed as described in P0. Three water flea generations were exposed to DEHP; i.e. P0, F1, and F2. However, F3 progeny were transferred into the normal medium without DEHP*

Data analysis

After Boxplot had reviewed the data, outliers or incorrectly detected water fleas had been eliminated. Only reliable data was used in the data analysis. The mean and standard deviation were used to express all data. Statistical tests were performed using

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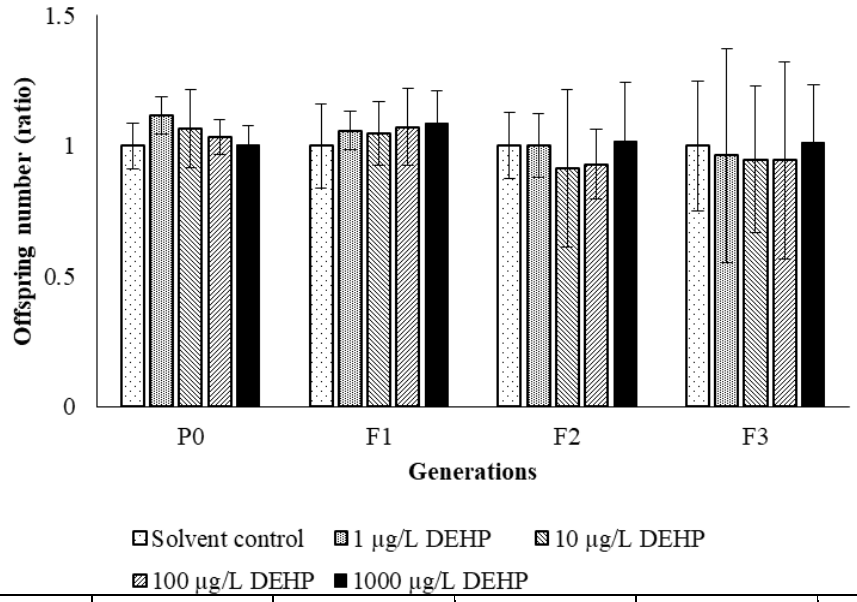
SPSS version 11.5. One-way ANOVA was used to analyze the statistical differences, and the significance threshold was set at 0.05. Kolmogorov-Smirnov (KS) test and Shapiro-Wilk test were applied to examine whether a dataset is normally distributed, and homogeneity of variance was assessed by the Levene test. Significant differences between the groups were examined using one-way ANOVA and post hoc test (Tukey HSD, LSD).

Results

Transgenerational effects on reproduction in a condition of DEHP exposure

The brood size of P0, F1, F2, and F3 of *M. macrocopa* was counted as total progeny. For brood size measurement, three independent biological replicates were performed with at least three *M. macrocopa* per group. *Figure 2* demonstrates that the offspring number (ratio) of P0, F1, and F2 generations in DEHP treatments (1, 10, 100, and 1000 µg/L) were not significantly different with solvent control. Furthermore, the results show that the offspring number (ratio) of F3 generation in the condition of no DEHP treatments was not significantly different from solvent control.



Offspring numbers (ratio)	Free-DEHP medium	1 µg/L DEHP	10 µg/L DEHP	100 µg/L DEHP	1000 µg/L DEHP
P0	1.00 ± 0.087	1.12 ± 0.183	1.07 ± 0.149	1.03 ± 0.066	1.00 ± 0.074
F1	1.00 ± 0.161	1.06 ± 0.073	1.05 ± 0.121	1.07 ± 0.148	1.09 ± 0.126
F2	1.00 ± 0.127	1.00 ± 0.122	0.91 ± 0.303	0.93 ± 0.135	1.02 ± 0.227
F3	1.00 ± 0.251	0.96 ± 0.409	0.95 ± 0.281	0.94 ± 0.378	1.01 ± 0.221

Figure 2. *Offspring numbers (ratio) of neonates of P0, F1, and F2 M. macrocopa exposed to 1, 10, 100, and 1000 µg/L DEHP with normal diets. In addition, F3 M. macrocopa was transferred into a free-DEHP medium with normal diets. The ratio was calculated from the offspring number of each DEHP treatment divided by the offspring number of solvent control. Also, the ratio was calculated within the same generation. Error bars are standard deviations. ANOVA and post hoc test were used to obtain the significant difference from solvent control (0.1% v/v DMSO). The outcome of the post hoc test (Tukey HSD, LSD) was not displayed on the plots because there is no significant difference*

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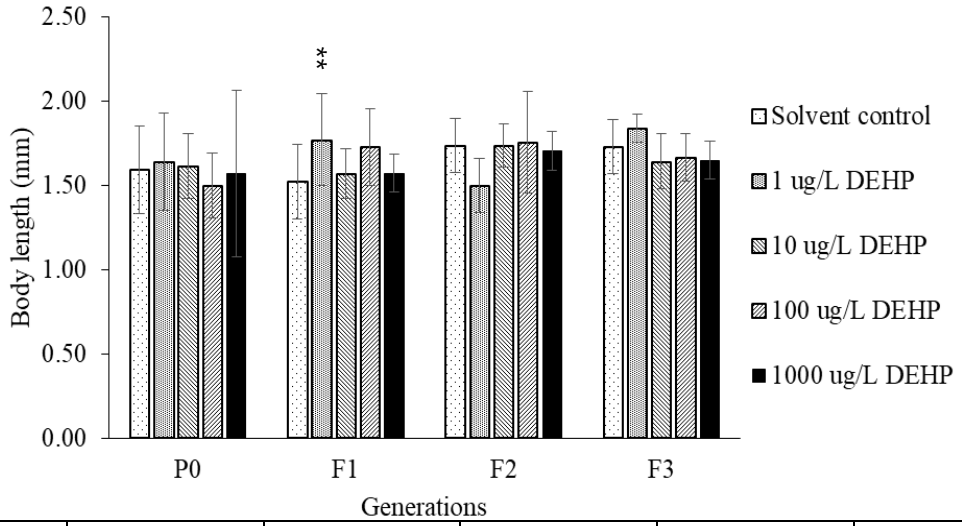
Transgenerational effects on growth in a condition of DEHP exposure

The body length of P0, F1, F2, and F3 *M. macrocopa* was measured in millimeters (mm). The body length of every *M. macrocopa* generation in solvent control ranged between 1.52 to 1.74 mm. The results showed that the body length of P0, F1, and F2 generations in DEHP treatments (1, 10, 100, and 1000 µg/L) was not significantly different from the solvent control (*Fig. 3*). In contrast, the body length of F1 *M. macrocopa* at 1 µg/L DEHP treatment significantly differed from solvent control. On the other hand, while F3 *M. macrocopa* was transferred into a free-DEHP medium with normal diets, the body length of this F3 generation was not significantly different from solvent control.



Body length (mm)	Free-DEHP medium	1 ug/L DEHP	10 ug/L DEHP	100 ug/L DEHP	1000 ug/L DEHP
P0	1.59 ± 0.260	1.64 ± 0.289	1.62 ± 0.194	1.50 ± 0.191	1.57 ± 0.492
F1	1.52 ± 0.222	1.77 ± 0.273**	1.57 ± 0.149	1.73 ± 0.227	1.57 ± 0.113
F2	1.74 ± 0.161	1.50 ± 0.160	1.74 ± 0.128	1.76 ± 0.301	1.70 ± 0.115
F3	1.73 ± 0.160	1.84 ± 0.081	1.64 ± 0.164	1.67 ± 0.139	1.65 ± 0.110

Figure 3. *Body length of P0, F1, F2 M. macrocopa exposed to 1, 10, 100, and 1000 µg/L DEHP with normal diets. In addition, F3 M. macrocopa was transferred into a free-DEHP medium with normal diets. Three independent biological replicates were performed with at least three M. macrocopa per group. M. macrocopa was observed by capturing the images under an optical microscope with a 5x objective lens and eyepiece camera. The captured image was then analyzed by image software ToupView 3.7 for body length. Error bars indicate standard deviations. ANOVA and post hoc tests (Tukey HSD, LSD) were used for significant difference tests from solvent control (** indicate $p < 0.01$)*

Discussion

In reality, more than one generation is affected by the pollution that we encounter every day. Pollutant toxicity experienced by the maternal generation may be passed on to the next generation. In order to investigate the transgenerational effects of DEHP exposure on the development and reproduction of *M. macrocopa*, this research was carried out.

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DEHP can be released into the environment in a variety of ways, one of which is through aquatic systems (Boonyatumanond et al., 2002; Clara et al., 2010; Fromme et al., 2002; Gao and Wen, 2016; Gevao et al., 2013; Long et al., 1998; Muenhor et al., 2018; Pham et al., 2011; Sirivithayapakorn and Limtrakul, 2007; Staples et al., 1997; Tan, 1995; Teil et al., 2006; Vitali et al., 1997; Wang et al., 2006; Wittassek et al., 2011; Yuan et al., 2002). Despite the fact that DEHP is primarily emitted into water bodies on a global scale, little research has been done on the effects. In various nations, DEHP concentrations in aquatic systems have been documented ranging from 0.33 to 97.8 g/L (Fromme et al., 2002; Long et al., 1998; Sirivithayapakorn and Limtrakul, 2007; Tan, 1995; Vitali et al., 1997; Yuan et al., 2002). Most findings indicated that DEHP exposure has adverse effects on organisms (Alfardan et al., 2018; ATSDR, 2002; Chen et al., 2015; Dombret et al., 2017; Grande et al., 2006; Hirai et al., 2015; Hopf et al., 2014; Jeddi et al., 2016; Li et al., 2015; Lyche, 2009; NTP-CERHR, 2006; Uren-Webster et al., 2010; Zanotelli et al., 2010). The purpose of this study was to determine whether DEHP contamination of water had a detrimental effect on the aquatic organism *M. macrocopa*, with a particular emphasis on the adverse influence on next generations. Throughout their entire lives and exposed for three generations, it was exposed to DEHP (1, 10, 100, 1000 μg/L) (*Fig. 1*). Following exposure to DEHP, growth and reproductive parameters were the indicators of this study.

Due to the persistence of DEHP, it was vital to evaluate the effects of long-term exposure to these compounds in aquatic settings. *M. macrocopa* was exposed to DEHP for whole lifespan for three generations (P0, F1, F2). Then, the F3 generation was removed from initial DEHP exposure to normal medium without DEHP. Offspring numbers (ratio) of neonates of P0, F1, and F2 *M. macrocopa* whose exposed to 1, 10, 100, and 1000 μg/L DEHP with normal diets showed no significant different with solvent control (*Fig. 2*). From the trial with 1 μg/L DEHP and normal diets, F1 generations were increased in body length (*Fig. 3*), indicating 1 μg/L DEHP can increase *M. macrocopa* growth. However, most results in *Figure 3* showed that the next generation was unaffected by 1, 10, 100, 1000 μg/L prolonged DEHP exposure. These findings reveal no significant decrease in longevity, growth, and reproduction of *M. macrocopa* when exposed to the environmental dose of DEHP and no significant difference between the blank control and the solvent control (0.1 v/v DMSO). When examined between solvent control and DEHP exposure, the indicators of growth, reproduction, and transgenerational effect were not significantly different (*Figs. 2* and *3*). These findings were in contrast to other reported studies that claimed DEHP had negative impacts on the growth and reproductive process (ATSDR, 2002; Chen et al., 2015; Grande et al., 2006; Jeddi et al., 2016; Li et al., 2015; Lyche, 2009; Uren-Webster et al., 2010; Zanotelli et al., 2009), acute toxicity of dibutyl phthalate (DBP) to freshwater *M. macrocopa* (Wang et al., 2009a), and chronic effects on survival and reproduction of butyl benzyl phthalate (BBP) to freshwater *M. macrocopa* observed in two successive generations of the cladoceran (Wang et al., 2011). This study suggested that antioxidant mechanism prevented transgenerational negative effects from continual DEHP exposure. As maternal *M. macrocopa* adapted to the contaminated environment, the negative effects were not passed on to their offspring. This outcome was consistent with research showing that DEHP could stimulate enzymatic antioxidant activity in aquatic species such as many kinds of zooplankton *D. Magna* (Wang et al., 2018), fish (Huang et al., 2015; Kaptaner et al., 2020; Mankidy et al., 2013; Qu et al., 2015; Yuan et al., 2017), pearl oyster (Xiang et al., 2017), and harlequin fly *Chironomus riparius*

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(Park and Kwak, 2012). Antioxidant defenses were likely activated, because DEHP cannot impact the production of vitellogenin and yolk bodies, therefore this study could not detect disturbed growth and reproduction during prolonged exposure to DEHP in three consecutive generations of *M. macrocopa* (*Figs. 2* and *3*). According to earlier findings, cellular pathology of the maternal (P0) ovarian cortex was observed under the transmission electron microscope (TEM), the majority of the data showed that exposure to 1000 g/L of DEHP over time resulted in normal adult ovarian cortex (Chaikritsadakarn, 2022; Chaikritsadakarn et al., 2024). Moreover, the explanation above was comparable to Park and Kwak (2012), showed that *C. riparius* metallothionein mRNA was upregulated in a situation where DEHP exposure had long-term impacts, and the level of vitellogenin mRNA was much higher. However, the superfamily of cysteine-rich proteins known as metallothionein (MT) was involved in the metabolism of metals, the removal of heavy metals from the body, and immunological responses such the defense against ionizing radiation and antioxidant defense (Wang et al., 2009b). The detoxification of DEHP may involve MT.

In addition to the antioxidant action, the environmental concentration of DEHP, and its degradation during the DEHP treatment process were considered. Equally to environmental concentration, DEHP concentration in this treatment ranged between 1 and 1000 μg/L, which was below the LC_{50} (4410 μg/L in *M. macrocopa*), where adverse effects were not noticed. Possibly, DEHP degraded during the DEHP treatment process, as that process was conducted in an environment with potentially present bacteria, all the processes involved in aerobic bacteria's degradation of phthalate esters were common for most microorganisms (Yuan et al., 2002).

The first step in the hydrolysis of phthalate esters into phthalic acid was the breaking of ester connections between alkyl chains and the aromatic ring. Consequently, for phthalate esters that biodegrade, the mineralization of phthalic acid via the dioxygenase-catalyzed pathway was crucial (Gao and Wen, 2016). Then, transgenerational negative effects of reproduction and growth from continual DEHP exposure were not displayed in *M. macrocopa*.

Conclusions

Although DEHP is principally released into water courses across the world, little in-depth research has been conducted on the environmental effects of its contamination on freshwater ecosystems. The present study is the first to examine the transgenerational effect of DEHP exposure on the growth and reproduction of *Moina macrocopa* (Crustacea: Cladocera). *M. macrocopa* is a zooplankton, an aquatic organism and an indigenous freshwater macroinvertebrate in Thailand. Hence, it may be a suitable animal to study the effects of DEHP on aquatic ecosystems. The findings reported here show that DEHP exposure to parental *M. macrocopa* had no toxic effects on growth, offspring number or capacity for reproduction. The exposure for the parental generation through F2 progeny did not lead to a concentration-dependent (1, 10, 100, 1000 μg/L DEHP) rising negative impact in subsequent generations, even with long-term exposure. Based on this evidence, the biotic potential and environmental resistance of *M. macrocopa* can be confirmed. This study also justifies further research of transgenerational effect of DEHP exposure on the growth and reproduction of other freshwater organisms with a focus on biodegradation properties and oxidative stress defense.

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Conflict of interests. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability. Data will be made available on request.

Ethical statement. Due to its status as an invertebrate under the 2015 Animal for Science Act, the water flea (*M. macrocopa*) was not listed for consideration by animal ethics committees. As a result, no permits or licenses were needed for this investigation.

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