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Transgenerational effects of the plasticizer di-2-ethylhexyl phthalate on survival, growth, and reproduction of *Daphnia magna*

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Abstract:

In this study, we conducted a chronic toxicity assessment of di-2-ethylhexyl phthalate (DEHP) on the life history traits of *Daphnia magna* across three generations. In the first generation, the neonates (called F0 *Daphnia*) were raised in a control environment (C, toxin-free medium) or in a medium containing DEHP at three concentrations 5, 50, and 500 µgl^{-1}, abbreviated as P5, P50, and P500, respectively. The offspring from the F0 control (called F1 *Daphnia*) were raised in toxin-free medium (denoted as C-C). However, the offspring from the P500 exposure were split into two groups: (i) the first group was raised in a toxin-free, control medium (denoted as P-C) and (ii) the second group was raised again in a medium containing 500 µgl^{-1} DEHP (denoted as P-P). The offspring from the F1 (called F2 *Daphnia*) were split again and treated in the same manner as F1, resulting in C-C-C, P-P-C, and P-P-P. The exposure time for each generation (F0, F1, F2) was 21 days. The survival and reproduction of *D. magna* over the three generations (F0, F1 and F2) were recorded daily during the 21 days of incubation. The body length of the animals in the F0 was measured by the end of incubation. The results showed that the survival rate of *D. magna* in the control and DEHP treatments was similar, while the DEHP strongly enhanced the reproduction of *D. magna* in the F0 and F1 generations. However, in the F2 generation, the survival rate for P-P-C and P-P-P was only 45-50% compared to the control. Consequently a much lower accumulative neonate proportion in DEHP treatment was found, around 50% compared to the control. The reduction in survivorship and reproduction of *D. magna* in the F2 generation and the smaller body length of the P500 treatment is a consequence of energy cost and trade-off under the chronic effects of DHEP. The results revealed that the population development of the micro-crustacean may lead to an extinction upon continuous exposure to high phthalate concentrations in natural water bodies. *In situ* monitoring on phthalates and zooplankton in aquatic ecosystems is suggested.

Keywords: **chronic effects, life traits, micro-crustacean, plastic additives.**

Classification number: **5.1**

Introduction

Global production of plastic materials has increased twenty-fold over the last fifty years, exceeding 300 million tonnes in 2015 [1]. Worldwide, a great amount of plastic waste is left unmanaged [2] and, more seriously, less than 5% of discarded plastic materials has been recovered [3]. Consequently, the continuous increase of plastic use over time has negative effects on the environment, especially water bodies. Plastics are known to contain a great number of additives, e.g., bisphenol A and phthalates, among others. Phthalates and their isoforms are among the most commonly used solvents in various industrial and consumer products, and the global production of phthalates is estimated to be between 6 and 8 million tons annually [4]. The existence of phthalates in the environment has been reported by many countries such as Finland, Denmark, Germany, Japan, China, Thailand, Poland, Sweden, and Italy [5]. While bisphenol A is known as both an oestrogen agonist and an androgen antagonist, impacting both reproduction and development in crustaceans and insects, phthalates have been shown to cause molecular and whole-organism effects in vertebrates and invertebrates [6]. Besides, phthalates desorbed from plastic have been known to accumulate in the gut of organisms resulting in disorder of biological processes such as endocrine disruption and behavioural alterations [7].

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Zooplankton have a central position in the food chain of aquatic ecosystems. Chemical substances leaching from many plastic products were shown to cause acute toxic effects (immobility) for *Daphnia magna*, with the 48 h-EC_{50} of leachates ranging from 5 to 80 g plastic material per L [8]. Giraudo, et al. (2015) found that the plasticizer Tris (2-butoxyethyl) phosphate (TBOEP) caused the mortality of 50% of the test *D. magna* within a 48 h exposure at a concentration of around 147 mgl^{-1} [9]. So far, there have only been a few investigations on the effects of plastic additives on freshwater micro-crustaceans such as *D. magna*. Plastic additives have also impacted gene transcription related to proteolysis, protein synthesis, and energy metabolism in *D. magna*. The plasticizer di-2-ethylhexyl phthalate (DEHP) at the concentration of 811 μgl^{-1}, significantly reduced the survivorship in *D. magna* after 21 days of treatment [10]. However, the same authors observed an impairment by DEHP exposure on genetic (RNA, DNA) and biochemical levels and hydrocarbon storage of the animal at a lower concentration, 158 μgl^{-1}, within 7 days of incubation. Recently, Wang, et al (2018) found that DEHP strongly influenced on the antioxidant and biotransformation enzyme activities in *D. magna* [11]. TBOEP at low concentration (10 μgl^{-1}) resulted in the reduction of body size (width and length), reproduction, and moulting in *D. magna* over three generational exposures [12].

Although the toxicity of plastic microspheres to several aquatic organisms has been tested and reported, the detrimental impacts of plastics and plasticizers on aquatic animals are underestimated [13]. The responses of aquatic animals and, in particular, zooplankton to microplastics and plastic additives upon long-term exposures are not yet fully understood [7, 14]. Therefore, in this study, we assessed the effects of DEHP at a concentration range of 5-500 μgl^{-1} on the life history traits of *D. magna* across three generations in laboratory conditions.

Materials and methods

The *Daphnia magna* (from Micro BioTest, Belgium) was raised in an ISO medium [15] and fed *ad libitum* with the live green alga *Chlorella* sp. and YTC, a rich nutrient mixture [16]. The alga *Chlorella* was cultured in a Z8 medium [17]. The animals were incubated under laboratory conditions at a temperature of 25±1^{0}C, light intensity of less than 1000 Lux, and a photo regime of 14 h light: 10 h dark [15, 18].

Di-2-ethylhexyl phthalate (DEHP, 99.5%), dissolved in MeOH at a concentration of 1000 mgl^{-1} (Aldrich Sigma), was used for the experiment. The stock was kept at 4^{0}C prior to the test implementation.

The experimental set up was conducted according to Dao, et al. (2010) and APHA (2012) with a minor modification [15, 18]. Briefly, the neonates of *D. magna* (<24 h old) were used for the chronic test and the experiment was conducted over 3 generations of *Daphnia*. In the first generation, the neonates (called F0 *Daphnia*) were raised in a control medium (denoted as C, a toxin-free medium) or in a medium containing DEHP at three concentrations: 5, 50, and 500 μgl^{-1}, abbreviated as P5, P50 and P500, respectively. The test concentrations of the phthalate in this study were based on the concentrations found in the environment, which is up to 460 μgl^{-1} [5]. The offspring from the F0 control (called F1 *Daphnia*) were raised in toxin-free medium (C-C). However, the offspring from the P500 were split into two groups: (i) the first group was raised in control medium (P-C), and (ii) the second group was raised in a medium containing DEHP (500 μgl^{-1}, P-P). The offspring from the second generation (called F2 *Daphnia*) were collected and split in the same manner as those from F1, resulting in C-C-C, P-P-C, and P-P-P (Fig. 1). We chose the offspring from the P500 for the transgenerational experiment because we believed that the P50 exposure would not cause significant effects on the life traits of the F1 and F2 *D. magna* generations because previous research found no observable effects on the bioindicators of *D. magna* (e.g. genetic and cellular levels, and life traits) were observed at a 158 μgl^{-1} concentration of DEHP [10]. However, a concentration of 811 μgl^{-1} DEHP impaired the survival, reproduction, and biochemical responses of *D. magna* on the 21st day of experiment [10]. Hence, we expected the P500 exposure might result in detrimental influences on *D. magna* in the F1 and F2 generations.

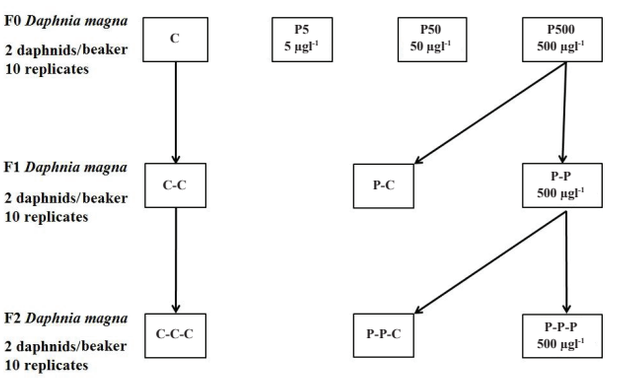

Fig. 1. Experimental setup. The first column shows the C, C-C, and C-C-C raised toxic-free medium exposures to *Daphnia* in the F0, F1, and F2 generations, respectively. The first row shows the C, P5, P50, and P500 exposures of *Daphnia* in F0 raised in 0, 5, 50, and 500 µg DEHP l^{-1}, respectively. P-C and P-P-C denote the exposures of *Daphnia* offspring of DEHP-exposed mothers in F0 and F1, respectively, raised in non-toxic medium. P-P and P-P-P denote exposures of *Daphnia* to DEHP-exposed mothers in F0 and F1, respectively, raised in a toxic medium of 500 µg DEHP l^{-1}.

In each generation and each treatment (control or DEHP exposure), 20 neonates were used and incubated in 10 glass beakers (2 neonate *Daphnia* per beaker, n=10; Fig. 1). The animals were fed and maintained in the laboratory conditions as mentioned above. The test medium and food were totally renewed 3 times per week and the incubation time for each treatment lasted 21 days. The life traits of the mother *D. magna,* including survival and reproduction, were recorded daily. By the end of experiment, the body size of the mother *Daphnia* in the F0 were measured on a microscope (Optika) coupled with a digital camera.

The Kruskal-Wallis test was applied in SigmaPlot (version 12.0) to determine the significant difference of the body size of F0 *D. magna* from the control and DEHP exposures.

Results and discussion

Effects of DEHP on survival of Daphnia magna

In the first generational experiment (F0), the survival rate of *D. magna* in the control and DEHP exposures was between 95% and 100% (Fig. 2A). The mortality of the animals in the second generational experiment (F1) was 85% (P-P), 95% (control), and 100% (P-C), as seen in Fig. 2B. In the third generational treatment (F2), the survival rate of the control was 95%, however, that of the P-P-C and P-P-P was 50% and 45%, respectively (Fig. 2C).

Seyoum and Pradhan (2019) reported that DEHP at concentrations up to 3900 μgl^{-1} did not result in any lethality or reduction of the hatching rate of *D. magna* within 48 h and 96 h, respectively, of incubation [4]. This record is similar to *D. magna* survival rate in control and DEHP treatments in our study. It has been evidenced that isoforms of phthalates (e.g. benzyl butyl phthalate, diethyl phthalate, mono-(2-ethylhexyl) phthalate, DEHP) at concentrations of 100 μgl^{-1} to 5 mgl^{-1} negatively influence the feeding behaviour of aquatic animals [7]. The values of the 48 h-LC_{50} of plastic additives and leachates on *D. magna* were reported to withstand quite a high range of the chemical and material concentrations, e.g., up to 147 mg TBOEP l^{-1} and 80 g plastic l^{-1} [8, 9]. Knowles, et al. (1987) found a reduction of genetic synthesis (DNA) and genetic ratio (RNA/DNA) in *D. magna* exposed to DEHP (158 μgl^{-1}) for 7 days [10]. Additionally, DEHP at concentrations of 60-100 μgl^{-1} significantly altered the activities of the antioxidant enzymes superoxide dismutase and catalase and the biotransformation enzyme glutathione S-transferase in *D. magna* within 2 days of exposure [11]. Hence, one might expect concentrations of 5-500 μgl^{-1} DEHP to cause adverse influences on biochemical and physiological responses on the F0 and F1 *D. magna* in our study, however, the impacts were apparently not strong enough to reduce the survivorship of the animals. Also, the survival rate of *D. magna* exposed 158 μgl^{-1} DEHP was similar to that of the control [10], which supports our observation in the F0 experiment (Fig. 2A). Giraudo, et al. (2015) reported that the plastic additive TBOEP did not cause mortality on *D. magna* at concentrations between 147-1470 μgl^{-1} [9]. Hence, the toxicity of DEHP and TBOEP to *D. magna* survival is comparable.

Differently, in the F2 generation, the *D. magna* pre-exposed to DEHP strongly reduced their survival within the first 4 days of incubation (Fig. 2C) regardless of being raised in a DEHP-containing medium or not. Phthalates were reported to impair the exposed animals on a molecular, cellular, and organ level [7]. The 811 μgl^{-1} DEHP exposure strongly reduced the total protein and glycogen content in *D. magna* after 21 days of exposure [10]. Therefore, an energy cost occurs when the animals are exposed to the toxin. The reduction of survival rate in the F2 generation in our study could be explained by: (i) there was already an energy cost and some impairment in the DEHP-exposed F1 *D. magna* and its offspring (F2) because DEHP could cause adverse effects on the development of the newborns [5, 10], and (ii) the impairment was only strong enough after 2 generational exposures to the DEHP. Hence, the F2 *D. magna* with prior disorder/damage would not be healthy enough to maintain normal life activities and die as a consequence. Thus, the toxicity of plasticizers to zooplankton should not be assessed within one generation, but by multiple generations.

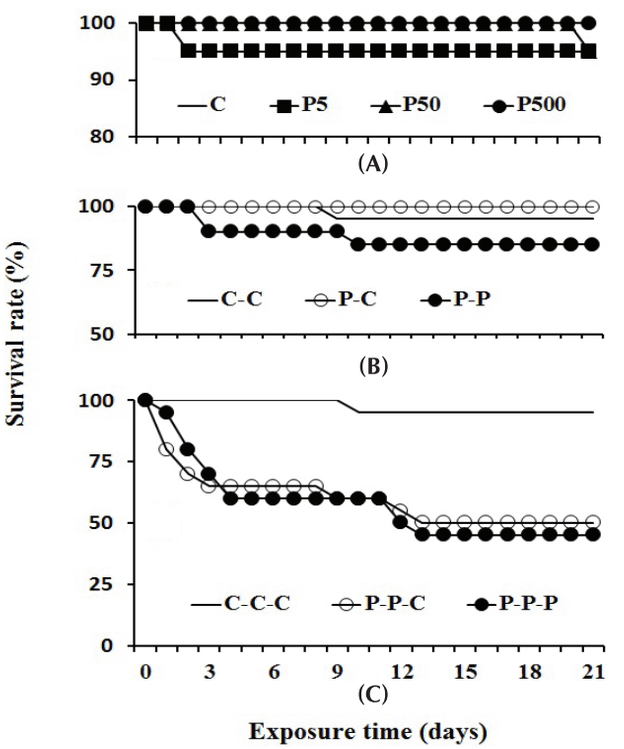

Fig. 2. Survival rate of the (A) first, (B) second, and (C) third generation of *Daphnia magna* exposed to DEHP. Abbreviations are the same as in Fig. 1.

Effects of DEHP on reproduction of Daphnia magna

The total neonates produced by the mother *D. magna* in the control, P5, P50, and P500 exposures were 545, 779, 1031, and 612, respectively. Therefore, in the F0 experiment, the total neonates of the P5, P50, and P500 relative to the control was 143%, 189%, and 112%, respectively (Fig. 3A). In the F1 experiment, the total neonates of the P-C and P-P relative to the control was 108% and 127%, respectively (Fig. 3B). Conversely, the total neonates of the P-P-C and P-P-P exposures relative to the control in the F2 experiment was 53 and 55%, respectively (Fig. 3C).

The plasticizer TBOEP (147-1470 μgl^{-1}) did not significantly alter the reproduction of *D. magna* during 3 weeks of treatment [9]. In contrast, DEHP at lower concentrations (e.g. 5 and 50 μgl^{-1}) resulted in a reproduction stimulation of the *D. magna* in the current study. Therefore, the influence of DEHP on *Daphnia* reproduction is much stronger than that of TBOEP.

Knowles, et al. (1987) proved that DEHP (from 158 μgl^{-1}) inhibited some biochemical components in *D. magna*. However, no significant impact on those components were observed when the animals were exposed to DEHP at concentrations up to 72 μgl^{-1} [10]. However, a DEHP concentration of 390 μgl^{-1} could increase the reproduction of *D. magna* up to 1.5 times compared to the control [4]. This helps to explain the enhancement of the total offspring in the DEHP-exposed mother *D. magna* compared to the control in the current study (Fig. 3A). Also, the remarkable increase of reproduction in the lower DEHP concentration exposures (P5 and P50) and slight increase in the reproduction of the higher DEHP concentration treatment (P500) is observed in our study (Fig. 3A). This may be explained by an energy cost of the high DEHP concentration treatment (P500 > 158 μgl^{-1}), which was reported elsewhere [10].

The reproduction of DEHP-treated *D. magna* in the F1 generation of our study was still a little higher than that of control. Phthalates and their isoforms are known to cause impairment of the reproduction of fish and aquatic mammals, including problems with fertility [5]. However, our results indicated oestrogen-like effects of the DEHP, i.e. a reproduction stimulation, on *D. magna,* as reported elsewhere [4]. It is likely that different species would have different responses to the same pollutants. Therefore, more investigations of this subject using phthalates are recommended to clarify the observed reproduction stimulation.

In the third generational exposure, F2, we found the total offspring in the pre-DEHP treated *D. magna* remarkably decreased, regardless if the animals were raised in a DEHP-containing medium or toxin-free medium (Fig. 3C). The fecundity of *D. magna* also significantly decreased, but the survival rate did not decrease after three generational exposures to the plasticizer TBOEP [12]. However, it is important to note that the 50% drop in total reproduction of the P-P-C and P-P-P exposures should be closely related to the total number of *D. magna* mothers in the experiment. Around 45-50% of the total number of *D. magna* mothers was lower in the P-P-C and P-P-P, starting from the 4th day to the end of experiment (Fig. 2C), and the accumulative neonates in the pre-DEHP incubations were reduced. Anyway, the population development of *D. magna* would continuously decrease in natural water bodies upon the presence of high phthalate concentrations for a long period of time. *In situ* monitoring of chemical concentrations and zooplankton population development is suggested for extrapolation in aquatic ecosystems.

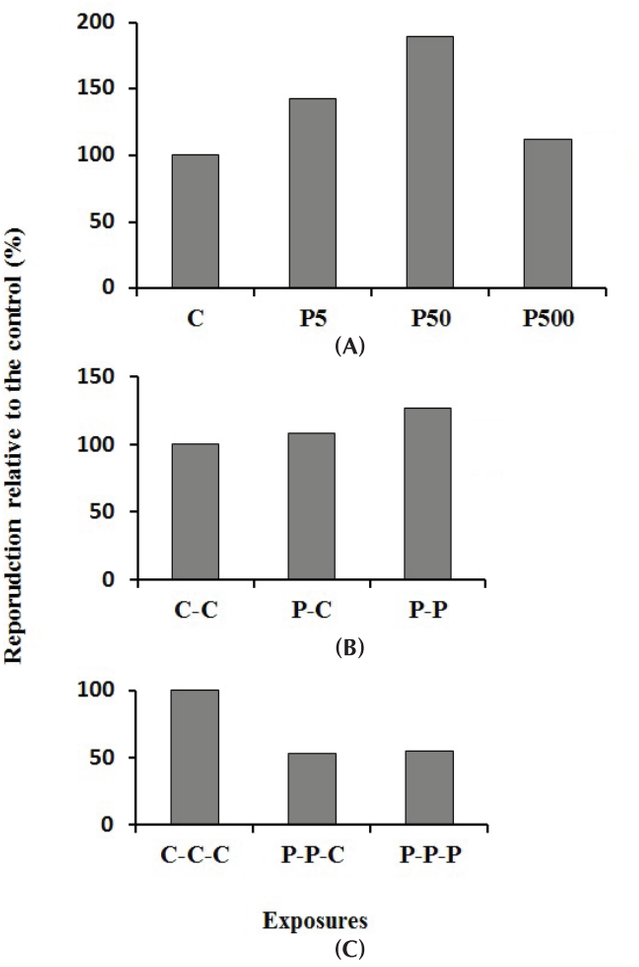

Fig. 3. Total neonates relative to control in the first (A), the second (B), and the third (C generations of *Daphnia magna* exposed to DEHP. Abbreviations as in the Fig. 1.

Effects of DEHP on body length of Daphnia magna

The body length of the mother *D. magna* at the age of 21 days in the control, P5, and P50 were similar, and ranged between 2883 and 2884 mm. However, the body length of the animal in the P500 exposure was 2580 mm, which is significantly shorter than that of the control (p=0.004, Kruskal-Wallis test; Fig. 4).

The similar body length measured of the *D. magna* in the control, 5 μgl^{-1}, and 50 μgl^{-1} DEHP concentrations in this study is in line with the previous observation by Giraudo, et al., (2015, 2017) testing with TBEOP [9, 12]. However, in an exposure to a higher DEHP concentration (500 μgl^{-1}), the *D. magna* was smaller size than in the control (Fig. 4), which is in line with a previous investigation of Seyoum and Pradhan (2019) [4]. The authors recorded a reduction of body length in *D. magna* exposed to around 390 μgl^{-1} of DEHP over the period of 14 days [4]. As previously mentioned, phthalates could impair aquatic animals at the genetic, cellular, tissue, and individual levels [7]. In the treatment with DEHP, it is believed that *D. magna* would be affected by the chemical. The animal could maintain their normal activities and deal with the biochemical and physiological alterations inside its body [10]. Then, the animal needs to balance its total energy for all of its life processes. Therefore, exposure to high concentrations of DEHP would lead to an energy cost in the *D. magna* that results in a trade-off between its growth and other activities. Maybe at low DEHP treatments (e.g. 5 and 50 μgl^{-1}), the *D. magna* could balance all processes and it could grow normally. However, at a higher chemical level incubation (500 μgl^{-1}), the animal has to face a trade-off that consequently slows down its development, hence, growing to smaller size than usual.

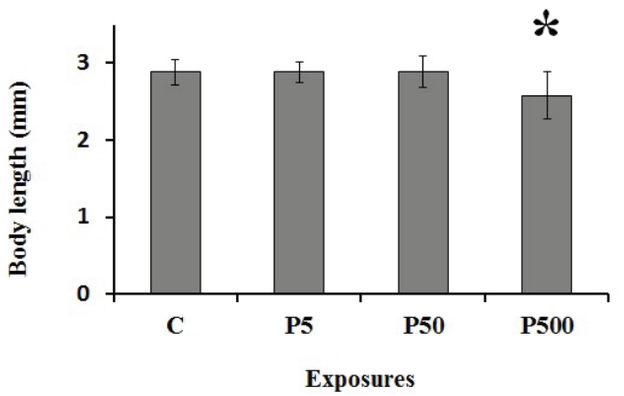

Fig. 4. Body length of the first generation of *Daphnia magna* exposed to DEHP at different concentrations. The asterisk indicates a significant difference of the body length between control and exposure (p=0.004, Kruskal-Wallis test). Abbreviations are the same as in Fig. 1.

Conclusions

This study, from the best of our knowledge, is the first to assess the effects of DEHP on the life history traits of *D. magna* across three generations. Survivorship of the animal exposed to the chemical was slightly reduced in the first and second generations. However, a high mortality proportion of 45-50% occurred in the DEHP exposures in the third generation. Acting as an endocrine disrupting compound, DEHP strongly stimulated the reproduction of *D. magna* in the first two generations (F0 and F1). However, the total accumulative offspring of *D. magna* was significantly reduced in the F2 generation, which is closely related to the survival rate of the pre-DEHP exposed mother *D. magna*. This shows that the population development of micro-crustacean may lead to an extinction upon continuous exposure to high phthalate concentrations in natural water bodies. The reduction in survivorship and reproduction of *D. magna* in the third generation and the smaller body length of the *D. magna* in the 500 μgl^{-1} DEHP treatment should be a consequence of energy cost and trade-off due to chronic effects of the chemical. Phthalates have been widely found in natural environment, but their toxicity to tropical aquatic animals has not been fully understood. Therefore, further investigations on the occurrence, distribution, and fate of phthalates, as well as their detrimental impacts on aquatic ecosystems, are highly suggested.

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