Effect of Dibutyl phthalate on antioxidant parameters and related gene expression in *Daphnia magna*

Tianyi Wang, Jie Wei, Li Wang, Yanan Lu, Qichen Zhang and Yuan Wang 1

Key Laboratory of Hydrobiology in Liaoning Province’s Universities, Dalian Ocean University, Dalian 116021, China.

1 Email: wangyuan@dlou.edu.cn

Abstract. Dibutyl phthalate (DBP) is a widespread environmental pollutant that poses a major threat to aquatic organisms and humans. However, the toxicological influences of DBP on zooplankton have not been completely researched. To determine the effect of DBP on antioxidant parameters and mRNA levels of antioxidant enzyme gene in *Daphnia magna*, *D. magna* were cultured with the sublethal concentrations of 0.5 or 2 mg/L DBP. We measured hydroxyl radicals (\(\cdot\)OH) scavenging and peroxidase (POD) activities of *D. magna* exposed to DBP. The expression levels of the two antioxidant enzyme genes glutathione S-transferases (GST) and catalase (CAT) in *Daphnia magna* were also analysed using quantitative real-time polymerase chain reaction. The results showed that the \(\cdot\)OH scavenging activity in *D. magna* exposed to DBP were increased at 24 h and decreased at 48 h. The POD activities in *D. magna* showed no obvious change under 0.5 mg/L DBP treatment, but were increased significantly at 2 mg/L DBP exposure. We observed significant increase in *gst* and *cat* mRNA levels in *D. magna* exposed to 0.5 mg/L DBP for 24 h, while the mRNA levels of the two genes in *D. magna* were reduced after 48 h DBP exposure. These data indicated that DBP-induced antioxidant defence systems were involved at the transcriptional level in *D. magna*.

1. Introduction

Phthalates (PAEs) are widely used as plastic additives in the industry. About 800 million pounds of PAEs were produced every year in the world and added to various commodities [1]. PAEs are easily released into the aquatic environment due to industrial, agricultural wastewater discharge and improper handling of plastics [2]. Dibutyl phthalate (DBP) is the most commonly detected PAEs in water environments around the world. High levels of DBP were reported in water, sediment and two lagoons (Epe and Lagos) of Nigeria [3] and in rivers and dams of the Venda region of South Africa [4]. In China, data on the mainstream of the Songhua River and its tributary environmental samples indicated that DBP concentrations had moderate or high risks to aquatic organisms [5]. The DBP concentration in Changjiang River Estuary and its adjacent area also exceeds the environmental risk levels (ERL) [6]. Therefore, the potential threat of DBP to aquatic organisms has attracted the attention of global environmental science researchers.

Previous ecological assessment studies focused on the adverse effects of DBP on aquatic animals [7, 8]. For example, low concentrations of DBP could damage the sperm of African clawed toads, resulting in embryonic deformities [9]. DBP could notably alter the peroxidase (POD) activity, reduce embryo hatchability and increase developmental malformations on abalone embryos [10]. *Daphnia magna* is not only commonly used as a model organism in aquatic ecotoxicological studies but often serves as an indicator species for pollution. Our previous study has shown that DBP stress treatment
was chronically toxic to *D. magna* and significantly inhibited the growth and reproduction of *D. magna* [11]. DBP induced over production of reactive oxygen species (ROS), the hydroxyl radicals (·OH) concentration of the red tide algae *Karenia brevis* showed a peak at 48 h exposed to DBP [12]. The activity of glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) of zebrafish embryos were all increased in a concentration-dependent manner after exposure to DBP [8]. DBP stimulated CAT activity in *Paralichthys olivaceus*, counteracting the oxidative stress [13]. Organisms often alter gene expression that lead to changes in antioxidant enzyme activity levels when exposed to stressful environments. In Daphnia species, recent studies have reported antioxidant enzymes gene expression, such as *cat*, *sod*, *gst* related genes transcript level changes, exposure to heavy metal [14, 15]. We recently reported that the *cat* and *gst* gene expression levels were notably reduced or increased upon Di-(2-ethylhexyl) Phthalate (DEHP) exposure in *D. magna* [16]. As *D. magna* could serve as a model for how aquatic species respond to toxicants, analyzing the influences of DBP on antioxidant parameters and gene expression patterns of related enzyme in *D. magna* may provide insights into how aquatic organisms respond to PAEs.

In the present study, we evaluated two antioxidant parameters ·OH scavenging and POD activities in *D. magna* cultured with the sublethal concentrations of 0.5 or 2 mg/L DBP. The changes in *cat* and *gst* expression levels were detected in *D. magna* exposed to sublethal concentrations of DBP using quantitative real-time polymerase chain reaction (qRT-PCR). These data provide useful information for understanding the toxicological responses of aquatic organisms to PAEs.

2. Materials and methods

2.1. Experimental materials

*D. magna* were collected from LiaoNing Key Laboratory of Dalian Ocean University. Animals were placed in glass beaker and grown in an illumination incubator with a 12 h light/12 h dark photoperiod and temperature at 20 ± 1°C (Themo 3744, Germany). Dechlorinated tap water was used as the culture medium, with total hardness 7.27 ± 0.2 mmol/L, alkalinity 12.47 ± 0.3 mmol/L, dissolved oxygen 7.96 ± 0.5 mg/L and pH 7.8 ± 0.2. Animals were fed daily with *Scenedesmus sp.* at concentration of 3×10⁵ - 4×10⁵ cell/mL.

DBP (CAS No: 84-74-2) was obtained from Sinopharm Chemical Reagent Co.Ltd (Shanghai, China). DBP were solubilized with acetone for the toxicity experiments. All chemicals used were of analytical grade.

2.2. Measurement of antioxidant parameters

According to preliminary experimental data and our previous research [11], the sublethal concentrations of 0.5 or 2 mg/L DBP were used to examine the effect of DBP on antioxidant parameters and related gene expression in *D. magna*. DBP concentrations (0.5 and 2 mg/L) and a blank and solvent control were set up in our experiment. 400 *D. magna* (> 96 h, length 2.67 ± 0.05 mm) were exposed to culture conditions as previously described. At 24 and 48 h, 200 living organisms were exposed to culture conditions as previously described. At 24 and 48 h, 200 living organisms were collected: 100 individuals were used to measure antioxidant parameters, and 100 individuals were used for mRNA levels detection. Each concentration obtained four replicates.

Samples were pooled in centrifuge tubes (1.5 mL) and immediately placed on ice. *D. magna* was homogenized with 10% (w/v) of PBS (0.1 M, pH 7.4), and the homogenates were centrifuged at 10,000 r/min for 10 min (4°C) (Eppendorf, Centrifuge 5804). The supernatants were then collected for determination of antioxidant parameters analysis. Protein content, ·OH scavenging and POD activities were measured using Nanjing Jiancheng Bioengineering assay kits (Nanjing, China). The absorbance value was evaluated using a microplate reader. Protein content was determined according to Bradford using bovine serum albumin as standard [17]. The Fenton reaction was adopted for the generation of ·OH [18]. The increase of ·OH during the hydrogen peroxide (H₂O₂) and Fenton reaction was indicative of ·OH activity. The value was detected at 550 nm. POD activity was detected using the method of Zhang FQ and Wang YS [19]. The result was expressed in units of POD activity per mg of
protein, where 1 U of POD is defined as the amount of enzyme decomposing 1 mmol of H₂O₂ per min. The value in absorbance at 470 nm was recorded.

2.3. qRT-PCR analyses

40 living *D. magna* in each tube were homogenized immediately on ice by a mortar in RNeasy solution (QIAGEN, USA). Total RNA from *D. magna* was extracted with TaKaRa MiniBEST Universal RNA Extraction Kit (TaKara, Japan). RNA integrity was analyzed on 1.2% agarose gels. Concentrations and purity of the total RNA were measured using a Scientific NanoDrop 2000 (NanoDrop Technologies Wilmington, USA). Reverse transcription was performed using the PrimeScript™ RT reagent Kit (Takara).

The cDNA synthesis reactions 20 μL were diluted into 100 mL RNase-free water. The diluted cDNA was used as template for 20 μL PCR reactions, containing forward and reverse gene-specific primer 0.8 μL, TransStart@Top Green qPCR Super Mix (2×) 10 μL, Passive Reference Dye II (50×) 0.4 μL, RNase-free water 6.8 μL, first strand cDNA 2 μL. qRT-PCR was conducted on StepOne®Plus (Applied Biosystems, Foster City, CA, USA) and the reactions were carried out on MicroAmp optical 96-well reaction plates (Applied Biosystems, Foster City, CA, USA). The amplification conditions were the following: initial denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, primer annealing at 54°C for 34 s with 40 cycles. Changes in gene expression were determined using the comparative CT (ΔΔCT) method was utilized to determine the relative target quantity. Primers for *β-actin*, *cat* and *gst* for *D. magna* were verified from Dominguez [20]. *β-actin* was used as control. The primer sequences used for qRT-PCR are shown in Table 1.

Table 1. Primer sequences used for the qRT-PCR study.

| Primer       | Primer sequence (5′-3′)        |
|--------------|-------------------------------|
| GST primer-F | CAACCGGTATGGCAAAGATG          |
| GST primer-R | CTAGACCGAAACGGETGAAA          |
| CAT Primer-F | CAGGATCATCGGAGTTAGTT          |
| CAT Primer-R | CTGAAGGCAAACCTGTCTACT         |
| *β*-actin-F  | CCTCCACCTCTTGGAGAAAT          |
| *β*-actin-R  | CAAGAATGAGGGCTGGAAGAG         |

2.4. Statistical analysis

Statistical analysis was performed using SPSS 21 and Excel 2007 statistical software package. Statistical data were regarded as mean values ± standard deviation (SD) in our studies. The LC₅₀ and the 95% confidence limits were calculated using Probit. Student’s *t*-test was adopted for the comparison between groups by Excel. A *p* value < 0.05 was statistically significant, whereas *p* < 0.01 was considered as extremely significant.

3. Results

3.1. Oxidative damage of DBP on *D. magna*

We treated *D. magna* with 0.5 or 2 mg/L DBP for 24 and 48 h and detected ·OH scavenging and POD activities (Figure 1-4). Upon 24 h exposure, there was no obvious change of ·OH scavenging activity in *D. magna* exposed to 0.5 mg/L DBP. However, we observed that ·OH scavenging activity was significantly increased after 2 mg/L DBP treatment (*p* < 0.01, Figure 1). As shown in Figure 2, ·OH scavenging activities were significantly decreased under 0.5 and 2 mg/L DBP treatment for 48 h (*p* < 0.05, *p* < 0.01, respectively). Upon 24 h exposure, POD activity in *D. magna* exposed to 0.5 mg/L DBP showed no significant change. POD activity was significantly increased in *D. magna* under 2 mg/L DBP treatment (*p* < 0.05, Figure 3). The activity of POD was also shown a significant increase in *D. magna* exposed to 2 mg/L DBP for 48 h (*p* < 0.01, Figure 4).
3.2. DBP exposure on cat mRNA expression of D. magna

To investigate whether DBP exposure modulates cat expression patterns in *D. magna*, we treated *D. magna* with 0.5 and 2 mg/L DBP for 24 and 48 h, and evaluated cat mRNA expression by qRT-PCR (Figure 5, 6). At 24 h, cat expression was significantly increased in *D. magna* exposed to 0.5 mg/L DBP ($p < 0.05$), while cat expression was significantly reduced in *D. magna* exposure to 2 mg/L DBP ($p < 0.01$, Figure 5). Comparison with control group, *D. magna* exposed to 0.5 and 2 mg/L DBP for 48 h, it showed significant reduction in cat mRNA expression ($p < 0.01$, Figure 6). We observed a concentration-dependent change in the expression of cat mRNA.
3.3. DBP exposure on gst mRNA expression of D. magna

To investigate whether DBP exposure modulates gene expression patterns of GST in D. magna, we treated D. magna exposed to 0.5 and 2 mg/L DBP for 24 and 48 h, and evaluated gst mRNA expression by qRT-PCR (Figure 7, 8). As showed in Figure 7, D. magna exposure to 0.5 mg/L DBP for 24 h resulted in a significant increase in gst mRNA level, while the gst expression showed a significant decrease in 2 mg/L DBP treatment (p < 0.01, p < 0.05, respectively). D. magna exposed to DBP for 48 h showed significant reduction in gst mRNA expression compared to the control (Figure 8). The inhibition of gst expression was more pronounced when D. magna were exposed to DBP for a prolonged period of time.

4. Discussions

Because of its indiscriminate pollution of aquatic organisms, DBP has been listed as an environmental priority pollutant by international organizations [21]. DBP can bio-accumulate and spread in the organism through the food chain of the ecosystem, causing irreversible damage to aquatic species [12, 13]. It has been reported that ROS was attacked in many aquatic organisms exposed to toxic levels of PAEs, and ROS production has been recognized as one of the consequences of toxicity response to PAEs [12, 22].
The ROS include free radicals such as ·OH, superoxide anion radicals (O2–) and non-free radicals such as singlet oxygen (1O2) and H2O2 [23]. Meanwhile, organisms are equipped with defence mechanism for repairing the ROS-induced damage. Antioxidant enzymes (such as SOD, POD, CAT, and GST) are the part of adaptive response mechanisms of pollution stress, which can remove free radicals to prevent oxidant damage [16, 24]. In the present study, the ·OH scavenging activity in D. magna showed a trend of increase at 24 h and decrease at 48 h. This indicated that the antioxidant mechanism of D. magna treated with DBP for 24 h can effectively detoxify ROS. However, after 48 h DBP exposure, the oxidative damage induced by DBP became severe, defence systems could be overwhelmed, resulting in inhibition of oxidative damage to body. It was consistent with previous study [25]. Combination with our work, it can be concluded that oxidative damage maybe one of the main factors to lead to body injury. POD and CAT are H2O2 removers that protects the body by hydrolyzing H2O2 into water and oxygen [24]. In the present study, POD activities were significantly increased under high concentration DBP treatment (2 mg/L), suggesting that DBP triggered oxidative stress. It was reported that POD contents on abalone embryos were essentially the same as the controls at the low-dose DBP levels of 0.05 and 0.2 μg/mL, while POD activities increased with DBP dose elevation (p < 0.05) [10]. This is consistent with our findings.

GST is a phase II detoxification enzyme and antioxidant that can resist oxidative stress as non-enzymatic carrier proteins for ligands [14, 26]. In the present study, the cat and gst mRNA expression could be induced by DBP, but the induction level depends on the strength and duration of exposure to stress [27]. We found that gst and cat mRNA in D. magna exposed to 0.5 mg/L DBP for 24 h showed significant increase while they showed significant reduction at 48 h (p < 0.01, p < 0.05). It is likely that D. magna under slight stress could induce a defensive response of antioxidant system. Activities of the antioxidant enzymes were activated, and increased in response to the oxidative challenges. The inhibitory effect became obvious, the gene expression levels were shown a notable reduction with prolong exposure. Upon 48 h DBP exposure, we observed significant reduction of gst and cat mRNA levels in D. magna exposed to 0.5 or 2 mg/L DBP compared to the control (p < 0.01). These result were consistent with the toxic effect of DEHP on gst in D. magna [16]. This indicated that D. magna under prolong DBP exposure suffered oxidative stress damage. The oxidative damage induced by DBP exceeds the body's antioxidant capacity, leading to a decrease in the expression level of antioxidant enzyme-related genes.

5. Conclusions
We conducted antioxidant parameters and transcriptional level analyses to establish the relationship between oxidative damage at the molecular level and DBP-induced stress in D. magna. Our results suggested that DBP could cause oxidative stress by inhibiting gst and cat expression. It was suggested that gene expression of antioxidants could be measured as pollution biomarkers in monitoring biological effects of contaminants.

Acknowledgement
This work was supported by the National Natural Science Fund of China (Grant number 41501535) and (Grant number 40806047), and Natural Science Fund of Liaoning Province (Grant number 20180550774).

References
[1] Zhang Y, Liang Q, Gao R, Hou H, Tan W, He X, Zhang H, Yu M, Ma L, Xi B 2015 PLOS One 10 e0137998
[2] Eggen RI, Hollender J, Joss A, Schärer M, Stamm C 2014 Environmental Science & Technology 48 7683-9
[3] Adeogun AO, Ibtor OR, Omogbemi ED, Chukwuka AV, Adegbola RA, Adewuyi GA, Arukwe A 2015 Marine Environmental Research 108 24-32
[4] Fatoki OS, Bornman M, Ravandhalala L, Chimuka L, Gente B, Adeniyi A 2010 Water Sa 36
117-125

[5] Wen Z, Huang X, Gao D, Liu G, Fang C, Shang Y, Du J, Zhao Y, Lv L, Song K 2018 *Environmental Science & Pollution Research* **25** 7688-7698

[6] Zhang ZM, Zhang HH, Zhang J, Wang QW, Yang GP 2017 *Science of the Total Environment* **619-620**, 93-102

[7] Staples CA, Adams WJ, Parkerton TF, Gorsuch JW, Biddinger GR, Reinert KH 2010 *Environmental Toxicology & Chemistry* **16** 875-891

[8] Xu H, Dong X, Zhang Z, Yang M, Wu X, Liu H, Lao Q, Li C 2015 *Fish & Shellfish Immunology* **45** 286

[9] Gardner ST, Wood AT, Lester R, Onkst PE, Burnham N, Perygin DH, Rayburn J 2016 *Journal of Toxicology & Environmental Health Part A* **79** 71-82

[10] Zhou J, Cai ZH, Xing KZ 2011 *Environmental Pollution* **159** 1114

[11] Wei J, Shen Q, Ban Y, Wang Y, Shen C, Wang T, Zhao W, Xie X 2018 *Bulletin of Environmental Contamination & Toxicology* **101** 214

[12] Li FM, Wu M, Yao Y, Zheng X, Zhao J, Wang ZY, Xing BS 2015 *Chemosphere* **132** 32-39

[13] Kang JC, Jee JH, Koo JG, Keum YH, Jo SG, Park KH 2010 *Ecotoxicology & Environmental Safety* **73** 1449-1455

[14] Kim H, Kim JS, Lee YM 2017 *Toxicology & Environmental Health Sciences* **9** 300-308

[15] Lyu K, Zhu X, Wang Q, Chen Y, Yang Z 2013 *Environmental Science & Technology* **47** 8887-8893

[16] Wang Y, Wang T, Ban Y, Shen C, Shen Q, Chai X, Zhao W, Wei J 2018 *Archives of Environmental Contamination & Toxicology* 1-12

[17] Bradford MM 1976 *Analytical biochemistry* **72** 248-254

[18] Gutteridge JMC, Rowley DA, Halliwell B 1981 *Biochemical Journal* **199** 263-265

[19] Zhang FQ, Wang YS, Lou ZP, Dong JD 2007 *Chemosphere* **67** 44-50

[20] Dominguez GA, Lohse SE, Torelli MD, Murphy CJ, Hamers RJ, Orr G, Klaper RD 2015 *Aquatic Toxicology* **162** 1-9

[21] Agus HH, Sümer S, Erkoç F 2015 *Environmental Monitoring & Assessment* **187** 423

[22] Zheng Q, Feng M, Dai Y 2013 *Environmental Toxicology & Pharmacology* **36** 741-749

[23] Gülçin I, Beydemir S, Hisar O 2005 *Acta Veterinaria Hungarica* **53** 4

[24] Zhao X, Gao Y, Qi M 2014 *Ecotoxicology* **23** 626

[25] Han X, Qingyun YU, Lian JU, Lingling LI, Xie M 2018 *Ocean Development & Management*

[26] Pothisalam G, Kumaresan V, Palanisamy R, Bhatt P, Kuppusamy T, Pasupuleti M, Arockiaraj J 2013 *Fish & Shellfish Immunology* **34** 1730-1731

[27] Lu Y, Zhang P, Li C, Su X, Jin C, Li Y, Xu Y, Li T 2013 *Fish & Shellfish Immunology* **34** 142-146