Di-(2-ethylhexyl) phthalate induced the growth inhibition and oxidative damage in the microalga *Chlorella vulgaris*

Chenchen Shen, Yuan Wang, Qi Shen, Li Wang, Yanan Lu, Xin Li and Jie Wei

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

1 Email: weijie@dlou.edu.cn

**Abstract.** Di-2-ethylhexyl phthalate (DEHP) poses a great threat to aquatic ecosystems, with known hazards to aquatic species. The ecotoxicological mechanism of DEHP in ocean microalgae is not yet known. In this study, we investigated growth inhibition, oxidative damage and antioxidant enzyme activities in *Chlorella vulgaris* under DEHP treatment. After 5-d exposure to DEHP ranging from 2 to 10 mg/L, the growth of *C. vulgaris* was significantly inhibited. The 96-h median effective concentration values (96h-EC$_{50}$) of DEHP for *C. vulgaris* were 6.02 mg/L. This growth inhibition was associated with DEHP-induced oxidative stress, as evidenced by elevated levels of intracellular malondialdehyde (MDA) and hydrogen peroxide. The results showed that DEHP reduced superoxide dismutase and glutathione peroxidise activities, increased hydrogen peroxide level and MDA content in a concentration-dependent way. These results indicated that DEHP could have biochemical and physiological toxic effects in *C. vulgaris*. These findings will help to understand the toxicity mechanisms of DEHP and the environmental risk assessment of primary producers of aquatic ecosystems.

**1. Introduction**

Phthalate (PAEs) are widely used as plasticizers in a variety of daily products. PAEs are bioaccumulative and toxic, difficult to degrade, persistent and ubiquitous in the aquatic environment. Di-2-ethylhexyl phthalate (DEHP) is most often detected PAEs in a variety of water sources[1-3]. The global annual production of DEHP is 5.5 million tons. The annual production of DEHP in China is about 3.05-3.4 million tons, accounting for about 60% of the global annual DEHP production[2, 3]. Although some studies have shown that DEHP can be biodegraded by microorganisms, DEHP still existed in marine and terrestrial ecosystems due to its high consumption and sustained release into the environment[1, 2]. Due to its negative impact on non-target organisms, DEHP is a highly interesting compound for environmental toxicology researchers.

Microalga plays an important role in the water ecosystem food chain. It is the foundation of the aquatic ecosystem, as the main producer, the main element of the structure and dynamics of the food web[4]. If microalgae are adversely affected by toxicants, the surrounding organisms may also be directly or indirectly affected by the lack of food[5]. The toxicity mechanism of DEHP on algae has been researched. The dinoflagellate *Alexandrium pacificum* was highly sensitive to DEHP, and even at low levels, the Chl *a* content was reduced by 80% after 24 h hours of incubation with 10 μg/L DEHP. DEHP significantly inhibited the biomass, the photosystem perturbation, and the photosynthetic activity of *A. pacificum*[6]. It was also described that DEHP notably decreased the growth contents...
of Chl a and Chl b in *Scenedesmus obliquu*[7]. But, there are relatively few studies on how DEHP induces growth inhibition and the mechanisms that lead to marine microalgal death.

Antioxidant defense systems in organisms were adversely affected when exposed to certain environmental contaminants, and antioxidant defense systems have been proposed as potential biomarkers for monitoring marine pollution[8]. It has been also confirmed that reactive oxygen species (ROS) were excessively produced in various aquatic organisms exposed to toxic levels of PAEs, and it was analyzed that ROS production was one of the consequences of organisms being affected by the toxicity of PAEs[3]. ROS can destroy macromolecular biomolecules such as proteins, DNA, lipids and other small molecules, thereby inhibiting the normal growth and reproduction of aquatic organisms and affecting the community structure of aquatic ecosystems[9]. A notable elevation in intracellular ROS levels was detected in algae exposed to DEHP and dimethyl phenol (DMP), indicating the oxidative stress caused by these pollutants[10]. Dibutyl phthalate (DBP) was found to promote oxidative damage in *Gymnodinium breve* due to elevated levels of ROS[11]. DEHP bio-accumulates in biological tissues and can alter or reduce the enzymatic reaction process, ultimately leading to cell death[12]. Antioxidant enzymes scavenge ROS and prevent oxidant damage. Glutathione S-transferases (GST) catalyzes the conversion of various electrophilic metabolites and glutathione to hydrophilic compounds. In this way, GST could promote the detoxification and excretion of harmful compounds[13]. Previous research literatures have revealed that exposure to DEHP caused lipid peroxidation and changed the antioxidant enzyme activity of aquatic organisms. DEHP exposure led to decreased catalase (CAT) and superoxide dismutase (SOD) activities in *Carassius auratus* brain and kidney tissues[14]. We recently reported that the cat and gst gene expression levels were notably reduced or increased upon DEHP exposure in *Daphnia magna*[15]. However, to date there has been no data on DEHP-induced oxidative stress associated with the marine microalga *Chlorella vulgaris*.

The aim of this study was to analyze the toxic effects of DEHP on the cell growth and antioxidant defense system of *C. vulgaris*. The data are helpful to assess the sensitivity of microalgal species to DEHP toxicity. The results of this study will also help to explore the ecotoxicity mechanism of PAEs on marine microalgae.

### 2. Materials and methods

#### 2.1. Test organism and culture conditions

*C. vulgaris* specimens were obtained from Dalian Ocean University of Liaoning Key Laboratory of Aquatic Biology and batch cultured in the original medium as recommended by OECD TG 201 guideline[16]. Toxicity tests were performed in 250 ml Erlenmeyer flasks containing 100 ml culture medium where exponentially growing cells was inoculated at an initial concentration of $1.2\times10^6$ cells/mL. The culture was incubated under light intensity of 100 IE m$^{-2}$ s$^{-1}$ produced by two 20 W white fluorescent lamps with 12:12 light–dark photoperiod at 22 ± 1°C. DEHP stock solution was prepared by dissolving 5.1 μL DEHP (CAS No.117-81-7) in50 μL acetone and stored under 4 °C. The stock was diluted to 500 mL with dechlorinated tap water before application and spiked into the culture at final concentrations of 2, 4, 6, 8 and 10 mg/L. No DEHP was added to the control. Each test was performed in four replicates. The cell density of algal culture was determined using a Neubauer haemocytometer. Specific growth rate was calculated according to the following formula:

$$X= (\ln N_1-\ln N_0)/(t_1-t_0)$$

Where $N_0$ and $N_1$ are the coenobia concentrations at the beginning of cultivation ($t_0$) and day $t_1$, respectively.

#### 2.2. Determination of hydrogen peroxide and malondialdehyde content

Algal material was powdered in liquid nitrogen and further homogenized in 50 mM sodium phosphate buffer (pH 6.0) containing sea sand. The extract was centrifuged at 10,000×g for 30 min, and the supernatant was used for hydrogen peroxide ($H_2O_2$), malondialdehyde (MDA) and antioxidant enzyme analysis. $H_2O_2$ can be reacted with chromogenic agent to produce a molybdenic acid-peroxide
complex. \( \text{H}_2\text{O}_2 \) was measured by monitoring the absorbance of the complex at 405 nm using a \( \text{H}_2\text{O}_2 \) assay kit[17]. MDA, the cytotoxic symbolic product of lipid peroxidation, was quantified colormetrically by its reaction with thiobarbituric acid[18]. MDA was detected by measurement the absorbance of the supernatant at 532 nm using a MDA assay kit. The lipid peroxidation level was expressed as \( \mu \text{mol MDA formed using an extinction coefficient of 155 mM}^{-1}\text{cm}^{-1} \).

2.3. Antioxidant enzyme assays
SOD activity was determined using the xanthine/xanthine oxidase/nitroblue tetrazolium system, in which the inhibition of cytochrome \( c \) reduction by SOD was measured by the reduction of nitroblue tetrazolium[19]. Glutathione peroxidase (GPx) activity was determined by measuring the oxidation of NADPH at 340 nm in a coupled assay with glutathione reductase[20]. GPx activity was determined by measuring the reduction of oxidized glutathione at 340 nm, as demonstrated by NADPH oxidation. All reagents were purchased from Nanjing Jiancheng Bioengineering Institute, China.

2.4. Statistical analysis
Data are expressed as the mean values of the four replicates with standard deviation. Student’s \( t \) test was performed for comparisons using Excel. A \( p \) value < 0.05 was regarded as statistically significant, whereas \( p < 0.01 \) was considered as extremely significant.

3. Results

3.1. Effects of DEHP on growth
The effect of DEHP on growth of \( C. \text{vulgaris} \) was shown in Figure 1. The growth of \( C. \text{vulgaris} \) in all DEHP-treated groups was significantly reduced from 1 to 5 d compared to the blank control. The toxic effect of DEHP on \( C. \text{vulgaris} \) was significantly correlated with its concentration, and the concentration of DEHP was negatively correlated with the growth of \( C. \text{vulgaris} \). At 5 day, the cell density of \( C. \text{vulgaris} \) was significantly reduced after treatment with 2, 4, 6, 8 and 10 mg/L DEHP respectively, which was 15.3%, 27.1%, 43.6%, 71.3% and 75.7% lower than the blank control. The \( EC_{50} \) of DEHP for \( C. \text{vulgaris} \) were 6.02 mg/L. These results indicated that the DEHP exposure significantly inhibited the growth of \( C. \text{vulgaris} \).

![Figure 1. Algal growth curves at different concentrations of DEHP.](image-url)
3.2. Intracellular $H_2O_2$ and MDA contents
The $H_2O_2$ content in $C. vulgaris$ was closely related to the concentration of DEHP stress (Figure 2). After 5 days of DEHP exposure, the $H_2O_2$ content of $C. vulgaris$ in each treatment group increased significantly from 279 to 652%. On the 5th day, the MDA content in $C. vulgaris$ increased significantly with increasing exposure concentration to DEHP (Figure 3), indicating that DEHP caused lipid peroxidation in the cells of $C. vulgaris$. High concentration (10 mg/L) of DEHP treatment resulted in a 652% increase in MDA content in $C. vulgaris$ on day 5 compared to the blank control.

3.3. Activities of antioxidant enzymes
The effect of DEHP on the activity of antioxidant enzymes SOD and GPx in $C. vulgaris$ showed a concentration-dependent way (Figure 4 and 5). SOD activity was stimulated by DEHP and was significantly higher than that of control in all tests after 5 d exposure. The GPx activity of $C. vulgaris$ generally decreased by 22.3–28.1% compared to the blank control on 5th d DEHP treatments.

**Figure 2.** Influences of different concentrations of DEHP on $H_2O_2$ contents of $C. vulgaris$.

**Figure 3.** Influences of different concentrations of DEHP on MDA contents of $C. vulgaris$.

**Figure 4.** SOD activities of $C. vulgaris$ cultured with different concentrations of DEHP.

**Figure 5.** GPx activities of $C. vulgaris$ cultured with different concentrations of DEHP.
4. Discussion

4.1. Effects of DEHP on growth

As an indicator of algae biomass under photosynthesis, cell density represents an overall parameter of cellular metabolism. The reduction of algal biomass could reflect the physiological state of algal cells [21]. In this study, growth inhibition experiment of microalgae clearly manifested DEHP was severely toxic. The EC$_{50}$ was recognized as the most acceptable indicator for assessing the toxicity of environmental pollutants and was widely used in many research literatures [22]. The 96 h EC$_{50}$ of DEHP for C. vulgaris was 6.02 mg/L. And the inhibitory effects on C. vulgaris cells growth increased with the increasing DEHP concentration from 2 to 10 mg/L (Figure1). A recent study on the toxic effects of DEHP indicated the acute toxicity of DEHP to cell abundance in marine dinoflagellates Gymnodinium breve [23]. According to Melin and Egnéus results showed a notable cell number reduce for the freshwater green algae Chlorella emersonii and the marine diatom Thalassioriap seudomona from 24 h of exposure to 1.8 mg/L of DBP[24]. In our study, C. vulgaris seemed to be highly sensitive to DEHP even at low concentration levels (2mg/L).

4.2. Oxidative damage in DEHP exposed C. vulgaris

ROS, H$_2$O$_2$, and MDA are widely utilized as biomarkers of environmental exposure, with a variety of studies reporting the effects of environmental organic pollutants, toxins, heavy metals and other chemicals on ROS content in aquatic organisms[25]. ROS are normal products of cellular metabolism, played important roles in cell signaling and various biochemical reactions, and are involved in the regulation of cell function. The intracellular ROS mainly include superoxide radical (O$_2^-$), hydroxyl radical (-OH), H$_2$O$_2$ and singlet oxygen (¹O$_2$)[9]. In microalgae, H$_2$O$_2$ are mostly produced by electron transport activities in the biochemical reactions of mitochondria, chloroplasts and plasma membrane under various environmental stresses (e.g., drought, salinity, and organic pollutants)[26]. Increased ROS may result in attack of cellular molecules, inducing oxidative damage. The change of MDA level is widely used as an indicator of oxidative injury to cells and tissues. Therefore, we also assessed changes in lipid peroxidation in algae by measuring the MDA content, which is considered an important form of lipid peroxidation induced by various toxins. The intracellular MDA level was positively correlated with H$_2$O$_2$ levels in C. vulgaris, confirming that the increased in MDA level was stress response induced by overproduction of H$_2$O$_2$ following DEHP exposure (Figure 2 and 3). Consequently accumulation of H$_2$O$_2$ in algal cells induces lipid peroxidation in membrane of cell and organelle (eg, mitochondria, chloroplasts), resulting in changes in cell membrane permeability and fluidity [27]. Zhao revealed that the increase in MDA content in C. pyrenoidosa caused by graphene oxide and multilayer graphene was related to the increased ROS caused by oxidative injury [28]. In addition, cell membrane damage may affect nutrient transport and metabolites of the cell membrane, leading to cell death caused by prolonged starvation [29]. These data indicated that DEHP could affect algae growth by causing membrane lipid peroxidation and oxidative injury in algae cells.

4.3. Antioxidant parameters in response to DEHP stress in C. vulgaris

It has been reported that ROS were excessively produced in various aquatic organisms exposed to toxic levels of PAEs, and ROS production was considered to be one of the consequences of toxicity to PAEs [8]. To scavenge increased H$_2$O$_2$ and MDA and mitigate oxidative damage due to free radicals, the antioxidative stress systems in algae may play principal defense roles by intercepting or reducing H$_2$O$_2$ production. Antioxidant enzymes including SOD, CAT, GST and GPx are essential components for reducing redundant ROS production, and are the first line of the antioxidant defense system in cells and organisms[30]. SOD can catalyze the dismutation reaction of superoxide anion to form H$_2$O$_2$. CAT catalyses the dismutation of H$_2$O$_2$ to oxygen and water. GPx catalyzes the reduction of H$_2$O$_2$ and organic hydroperoxides with reduced glutathione[9]. We found that SOD activity was significantly decreased in C. vulgaris exposed to lower concentrations of DEHP for 5 d; the inhibitory effect
became obvious at higher concentrations. In the present study, SOD and GPx activities were significantly correlated with MDA content in algae (Figure 4 and 5). The decrease in antioxidant enzymes SOD and GPx activity at various concentrations after 5 d of DEDP exposure may be associated with increased ROS production. Under severe stress environment or prolonged stress, the excess amount of radicals generated by DEHP exposure exceeded the eliminating ability of antioxidant enzyme [31]. DEHP exposure led to failure of the antioxidant defence system of microalgae cells, resulting in high levels of lipid peroxidation in *C. vulgaris*, consistent with previous studies[32]. Antioxidant enzymes activities, \( \text{H}_2\text{O}_2 \) and MDA content depended on the level of DEHP-induced stress and contact time.

5. Conclusions
DEHP exposure had a significant inhibitory effect on the growth of marine microalgae *C. vulgaris*. In addition, DEHP-induced changes in intracellular \( \text{H}_2\text{O}_2 \) content were positively correlated with MDA level. Data analysis showed that DEHP stress caused lipid peroxidation and oxidative injury in marine microalgae cells. DEHP stress caused the oxidative injury of microalgae to exceed the antioxidant capacity of the cells. As the stress time prolonged, the activity of antioxidant enzymes such as SOD and GPx decreased. Based on these data, a large amount of DEHP released into the water may pose a serious ecological risk to algae or other aquatic organisms. This study will help to understand the toxicity mechanisms of PAEs to aquatic organisms and to assess the environmental risks of PAEs to primary producers of aquatic ecosystems. However, further investigations are needed on the bioaccumulation, biomagnification and degradation effects of PAEs in aquatic food webs.

Acknowledgements
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] Hammad K M and Jung J Y 2008 *Chemosphere* 72(4) 690-696
[2] Zhan Y, et al 2016 *Environmental Science & Technology* 50(5) 2450-2458
[3] Zhang C, et al 2010 *Aquatic Toxicology* 203 172-178
[4] Liu Y, et al. 2018 *Chemosphere* 80(5) 592-599
[5] Chi J, et al. 2007 *Journal of Environmental Science and Health Part A* 42(2) 179-183
[6] M'Rabet C, et al. 2018 *Marine Pollution Bulletin* 126(11) 241-249
[7] Wang L, et al. 2010 *In International Conference on Bioinformatics and Biomedical Engineering*
[8] Zheng Q Feng M and Dai Y 2013 *Environmental Toxicology & Pharmacology* 36(3) 741-749
[9] Rachana S, et al. 2016 *Frontiers in Plant Science* 7
[10] Sjollem A B. et al. 2016 *Aquatic toxicology* 170 259-261
[11] Bie C C, et al. 2012 *Environmental Science* 33(2) 442
[12] Yuan L, et al. 2017 *Comparative Biochemistry & Physiology Part C Toxicology & Pharmacology* 202 79-84
[13] Lu Y, et al. 2013 *Fish & shellfish immunology* 34(1) 142-146
[14] Zhang G S 2014 *Journal of Safety & Environment*
[15] Wang Y, et al. 2018 *Archives of Environmental Contamination & Toxicology* 1-12
[16] Chemistry Test 2006 *Oecd Guidelines for the Testing of Chemicals* 1 1-1(1)
[17] Tan W J, et al. 2006 *Journal of Southeast University*
[18] Davey M W, et al. 2005 *Analytical Biochemistry* 347(2) 201-207
[19] Crapo J, et al. 1978 *A journal of technical methods and pathology* 39(6) 640-653
[20] Flohé L and Günzler W A 1984 *Elsevier* 114-120
[21] Chen Z, et al. 2016 *Environmental Science & Pollution Research* 23(18) 17910-17918
[22] Tugcu G, Ertürk M D and Saçan M T 2017 *Journal of Hazardous Materials* 339 122
[23] Wilson W B , et al. 1978 Bull Environ Contam Toxicol 20(1) 149-154
[24] Acey R, et al. 1987 Bulletin of Environmental Contamination & Toxicology 39(1) 1-6
[25] Li F M, et al. 2015 Chemosphere 132 32-39
[26] Gomes T, et al. 2017 Aquatic Toxicology 183 1-10
[27] Van d P J , et al. 2015 Chemical Science 7(1) 489-498
[28] Zhao J, et al. 2016 Water Research 111 18-27
[29] Zeyons O l , et al. 2009 Nanotoxicology 3(4) 284-295
[30] Ken C F, et al. 2005 Journal of Agricultural & Food Chemistry 53(5) 1470-4
[31] Gui-sheng Z 2014 Journal of Safety and Environment 5 075
[32] Oliveira C, et al. 2012 Aquatic Toxicology 124-125 209-216