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# Joint Toxicity of Two Phthalates with Waterborne Copper to *Daphnia magna* and *Photobacterium phosphoreum*

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**Abstract** Di-*n*-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) are two widely used phthalates, while Cu(II) is a common valence state of copper. They have been ubiquitously detected in the aquatic environment, but information on their joint toxicity to aquatic organisms is scarce. In this study, we evaluated the combined effects of copper and these two phthalates to *Daphnia magna* and *Photobacterium phosphoreum* by quantifying the acute toxicity expressed by the $EC_{50}$ (the concentration causing 50 % of maximal effect) value. The toxicity order was DEHP + Cu(II) > DBP + Cu(II) > Cu(II) > DEHP > DBP for both test species. Antagonism effects were found in the joint toxicity of Cu(II) combined with DBP or DEHP using the toxic unit method. These findings have important implications in environmental risk assessment for phthalates in the aquatic environment in the presence of heavy metals.

**Keywords** Phthalates · Copper · Joint toxicity · *Daphnia magna* · *Photobacterium phosphoreum*

Phthalic acid esters (PAEs), or phthalates, are typical plasticizers and solvents for industry and daily life uses. They help enhance the flexibility of plastics, but are not chemically bonded to plastics, so they are inclined to release from plastics and enter into the ecosystem (Teuten et al. 2009). The wide use of PAEs has resulted in their worldwide occurrence as pollutants (Chen et al. 2008). PAEs have been classified as potential toxicants since they can alter reproductive development of aquatic invertebrates and terricolous mammals (Liu et al. 2009; Martino-Andrade and Chahoud 2010).

Di-*n*-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) are two commonly used PAEs. Due to their high production and extensive application, DBP and DEHP are commonly found in wastewater and industrial sewage sludge. In China, the Environmental Quality Standard for Surface Water (GB_3838-2002) stipulates the upper limit of DBP and DEHP as 1 and 4 μg/L, respectively. A recent study indicated that the concentration of DEHP was 78 μg/L in Bohai sea (in Tianjin, China), and DBP was also found at a relatively high concentration, 61 μg/L, in Qinghe River (in Beijing, China) (Wu et al. 2013). DBP and DEHP are teratogenic, mutagenic and carcinogenic, and the Environmental Protection Agency of the United States (U.S. EPA) has classified them as priority pollutants (Yin et al. 2003). These two PAEs exhibited toxicity to the fish, *Carassius auratus* (Huang et al. 2015).

Many studies have been performed on PAE toxicity toward terrestrial species or fish, but they have largely been limited to a single phthalate or a series of PAEs (Seo et al. 2004; Wang et al. 2012; Zheng et al. 2013). Little information exists on the joint effect of phthalates with heavy metal ions. Therefore, there is no scientific basis to assess the toxicity of PAEs when they coexist with metal ions in the aquatic environment, a scenario that is common in a contaminated environment. Phthalates and copper were found to coexist in the sediment of Yellow River (Xu et al. 2007). Copper is a heavy metal that is extensively used in industry, and it is commonly found in sewage and natural water bodies. Heavy metals in the aquatic environment are

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known to cause several health problems to animals and human beings (Dural et al. 2007).

The toxic unit method has been used to determine the effect of joint toxicity (synergistic, additive, antagonistic and independent). It was first introduced by Marking and Mauck (1975), and has been widely used to determine the effect of joint toxicity (Playle and Richard 2004; Khan et al. 2012).

The objective of this study was to assess the joint toxicity between two PAEs and copper by measuring the $EC_{50}$ (the concentration causing 50 % maximal effect) value of two indicator aquatic organisms: *Daphnia magna* and *Photobacterium phosphoreum* (T3 mutation). Both organisms are readily cultured in the laboratory, commonly used in aquatic toxicology, and respectively represent aquatic plankton and microorganism (Adema 1978; Brouwer et al. 1990). The joint toxicity effect was determined by the toxic unit approach.

## Materials and Methods

DBP (CAS No. 84-74-2) and DEHP (CAS No. 117-81-7) were purchased from Lingfeng Chemical Reagent Co. Ltd. (Shanghai, CN) and Sinopharm Chemical Reagent Co. Ltd. (Shanghai, CN), respectively. They were dissolved in dimethyl sulfoxide (DMSO) to obtain different experimental concentrations. Copper sulfate ($CuSO_4$), acquired from Xiaoshan Chemical Reagent Co. Ltd. (Hangzhou, CN), was dissolved in ultrapure water as stock solution. The maximum water solubility limits for DBP and DEHP are 11.2 and 0.01 mg/L, respectively (Metcalf et al. 1973). DMSO was used as a solvent carrier in this study. The maximum concentration of DMSO used in study of *P. phosphoreum* was 1.56 g/L. The DMSO concentration in *D. magna* experiment was below 660 mg/L. All chemicals used were of analytical grade.

Freeze-dried powder of *P. phosphoreum* (a luminescent marine bacterium) was obtained from the Institute of Soil Science, Chinese Academy of Sciences (Nanjing, CN). After injection of 0.5 mL of cold sterilized 2.0 % NaCl solution into a vial containing 0.5 g of freeze-dried powder, the solution was mixed thoroughly by shaking for 2 min. Then, 10 μL of the revived bacterial liquid was diluted with 2 mL of 3.0 % NaCl solution to serve as the working bacterial suspension for subsequent tests described below.

The first generation of *D. magna* was supplied by the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences (Beijing, CN). Tap water that had been passed through activated carbon and aerated for more than 48 h was used as culture water. Parent fleas were kept in the culture water (pH 7.25 ± 0.25) in a 14 h light/10 h dark cycle at 20°C, and were fed daily with green algae, *Scenedesmus obliquus*. Juvenile stage fleas that had undergone three generations of parthenogenesis (6–24 h old) were used in the experiment. The above operation complies with the National Standard Method of China (Water quality—Determination of the acute toxicity of substance to *Daphnia* (*Daphnia magna* Straus). GB/T 13266-1991).

The test of acute toxicity to *P. phosphoreum* was operated following the National Standard Method of China (Water quality—Determination of the acute toxicity—Luminescent bacteria test. GB/T 15441-1995). The working solution was prepared by serially diluting the stock solution of DBP, DEHP or copper sulfate ($CuSO_4$) with 3.0 % NaCl solution.

All the concentrations of test chemicals are nominal. In order to verify the actual concentrations, the copper sulfate ($CuSO_4$) solutions were measured by atomic absorption spectrophotometry using a Sollar M6 instrument (Thermo Corp., Waltham, MA, USA). The minimum detection limit for Cd was 0.067 μg/L. PAE solutions were measured by high performance liquid chromatography (Agilent Technologies Inc. Santa Clara, CA, USA). The optimum condition for HPLC analysis was achieved with acetonitrile/water (30: 70, v/v), UV detection wavelength of 224 nm, and a flow rate of 1 mL/min. The minimum detection limits of this method were 0.10 and 0.25 μg/L for DBP and DEHP, respectively. The linearity of standard curves was above 0.9998. The recoveries of spiked test chemicals were 88 %–107 %.

In the single toxicity experiment, exposure concentrations were set at 8 concentration gradients for each toxicant [0, 64, 96, 128, 160, 200, 300, 400 mg/L for DBP and DEHP; and 0, 4, 5, 6, 8, 9, 10, 11 mg/L for Cu(II)]. The concentration that caused a 50 percent bioluminescence inhibition was estimated as $EC_{50}$. The $EC_{50}$ value was calculated based on linear curve fitting between bioluminescence inhibition and the chemical concentration.

The experiment was performed in octuplicate in a black flat-bottom 96-well (8 rows × 12 columns) microplate (Thermo Corp.). The first two columns were used to test the sensitivity of the *P. phosphoreum*: each well in the first column was filled with 180 μL of 3 % NaCl solution as the blank control group, while an equal volume of $HgCl_2$ standard solution (0.1 mg/L) was added into each of the eight wells in the second column to serve as a positive control group. Similarly, 180 μL from the serial dilutions of the test chemical in the order of increasing concentration were injected into the wells of the remaining columns, each concentration taking 8 wells in one column. Then, 20 μL of the working bacterial suspension was added into each test well to achieve a test volume of 200 μL. Bioluminescence of the treatments and controls was determined on a Tecan

Infinite 200® PRO multimode microplate reader (Tecan, Männedorf, CH) after 15 min exposure at 25°C. In the joint toxicity experiment, the mixtures were prepared by mixing two chemicals at the same fractions of their respective $EC_{50}$. The concentrations of DBP and Cu(II) were 0, 0.25a, 0.5a, 0.625a, 0.75a, 0.875a and a (a was 390 mg/L for DBP and 7 mg/L for Cu(II), respectively, based on their $EC_{50}$ values); while in the mixtures of DEHP and Cu(II), their concentrations were 0, 0.2b, 0.4b, 0.6b, 0.8b, b and 1.2b [b was 302 mg/L for DEHP and 7 mg/L for Cu(II)].

The acute toxicity of the chemical-spiked samples to *D. magna* was determined following the National Standard Method of China (GB/T 13266-1991). According to preliminary experiments, eight concentrations [0, 0.1, 0.3, 0.6, 1, 2, 3, 4 mg/L for DEHP; 0, 0.6, 1.8, 3.6, 6, 12, 18, 24 mg/L for DBP; and 0, 0.025, 0.075, 0.15, 0.25, 0.5, 0.75, 1 mg/L for Cu(II)] were used to determine $EC_{50}$. Beakers were used as test vessels and 10 *D. magna* were added into 100 mL test solution. The vessels were placed at 20 ± 2°C with a 14 h light/10 h dark photoperiod in an illuminating incubator. Each experiment was replicated three times. No food was provided during the experimental periods. After 24 h exposure, the number of immobilized *D. magna* was recorded. For joint toxicity experiment, DBP or DEHP was mixed with Cu(II) at the same fractions of their respective $EC_{50}$ value tested as single toxicant. The concentrations of mixtures of DBP and Cu(II) were 0, 0.4c, 0.8c, c, 1.2c, 1.6c, 2c [c was 8 mg/L for DBP and 0.14 mg/L for Cu(II) according to the $EC_{50}$ of DBP and Cu(II)]; while the concentrations of DEHP and Cu(II) were 0, 0.4d, 0.8d, d, 1.2d, 1.6d, 2d [d was 2 mg/L for DEHP and 0.14 mg/L for Cu(II)].

The toxic unit approach was applied where: (1) $TU_i = C_i/EC_{50i}$ and $TU_i$ was the toxic unit for component $i$ in the mixture, $C_i$ was the concentration of component i when the mixture caused a 50 percent bioluminescence or *D. magna* immobilization, and $EC_{50i}$ was the $EC_{50}$ of compound i); and (2) $M = \sum_{i=1}^{n} TU_i = \frac{C_1}{EC50_1} + \frac{C_2}{EC50_2} + \cdots + \frac{C_n}{EC50_n}$. The form of joint toxicity can be determined by the value of $M$: a synergistic effect happens when $M < 1$; an additive effect occurs when $M = 1$; and an antagonistic effect takes place when $M > 1$. In certain cases, an independent effect may happen when the toxic unit of one chemical approaches to 1 ($TU_i = 1$), in which case the toxicity is primarily casued by one component in the mixture) (Marking and Mauck 1975).

## Results and Discussion

It has been reported that DMSO has no effect on toxicity of organic chemicals in terms of the $EC_{50}$ values when its concentration is no more than 0.20 mol/L or 15.6 % mass fraction (Dong et al. 2013). The concentration of DMSO, used as a solvent carrier for dissolving PAEs in water in this study, was below 0.2 mol/L, and thus was not considered to have caused any adverse effects. The nominal and measured concentrations of DBP, DEHP and Cu(II) are presented in Tables 1, 2, 3, 4, 5 and 6.

The response curves for *P. phosphoreum* toxicity tests are shown in Fig. 1. $EC_{50}$ values were calculated using the fitted equations shown in the figures. The abscissa is exposure concentration, and the ordinate is inhibition rate. According to the fitted equations in the figures, the value of $EC_{50}$ can be calculated: 390 mg/L for DBP, 302.5 mg/L for DEHP and 6.7 mg/L for Cu(II). For the joint toxicity experiment of DBP and Cu(II), the concentrations of DBP and Cu(II) when 50 % inhibition occurred were 378 mg/L and 6.5 mg/L, respectively. In the mixture of DEHP and Cu(II), 50 % inhibiting concentrations were 275 mg/L for DEHP and 6.1 mg/L for Cu(II). Based on the toxic unit approach, it was calculated that

$$M_a = \frac{378}{390} + \frac{6.5}{6.7} = 1.94 > 1,$$
$$M_b = \frac{275}{302.5} + \frac{6.1}{6.7} = 1.82 > 1$$

$M_a$ is the $M$ value of DBP combined with Cu for *P. phosphoreum*, $M_b$ is the $M$ value of DEHP and Cu for *P. phosphoreum*. Therefore, the effect of joint toxicity of DBP and DEHP combined with Cu(II) to *P. phosphoreum* was antagonism.

It was shown that, when the applied concentration of DMSO was below 660 mg/L (mass fraction 6.6 ‰), it had little effect on *D. magna* and can be used as solubilizing assistant (Haap et al. 2008). The use of DMSO in this experiment of *D. magna* was below 100 mg/L, and was not considered to have resulted in any adverse effects. The results are shown in Fig. 2. According to the linear equations in Fig. 2, the $EC_{50}$ values were calculated for DBP, DEHP and Cu(II) as 8.0, 2.1 and 0.14 mg/L, respectively. In the joint toxicity experiments with DBP and Cu(II), the concentrations of DBP and Cu(II) that caused 50 % immobilization of *D. magna* were 7.36 and 0.129 mg/L, respectively. Meanwhile, in the test of the mixtures of DEHP and Cu(II), $C_{DEHP}$ was 1.764 m/L, and $C_{Cu(II)}$ was 0.118 mg/L. According to the toxic unit approach, it can be calculated that

$$M_c = \frac{7.36}{8} + \frac{0.129}{0.14} = 1.84 > 1,$$
$$M_d = \frac{1.764}{2.1} + \frac{0.118}{0.14} = 1.68 > 1$$

$M_c$ is the $M$ value for DBP and Cu for *D. magna*, and $M_d$ is the $M$ value for DHEP combined with Cu for *D. magna*. Therefore, the nature of the joint toxic effect when either DBP or DEHP was combined with Cu(II) was antagonism.

For *P. phosphoreum*, $EC_{50}$ values were relatively high, suggesting that the endpoint was relatively insensitive to

**Table 1** The nominal and measured concentrations of DBP in single exposure solutions (mg/L)

| Nominal | 0.6 | 1.8 | 3.6 | 6 | 12 | 18 | 24 |
|---|---|---|---|---|---|---|---|
| Measured | 0.58 ± 0.03 | 1.81 ± 0.01 | 3.67 ± 0.03 | 6.07 ± 0.03 | 12.86 ± 0.09 | 17.47 ± 0.11 | 23.57 ± 0.63 |
| Nominal | 64 | 96 | 128 | 160 | 200 | 300 | 400 |
| Measured | 65.15 ± 0.87 | 96.63 ± 1.54 | 126.12 ± 2.76 | 162.83 ± 2.63 | 196.79 ± 2.83 | 293.51 ± 3.69 | 391.17 ± 5.74 |

**Table 2** The nominal and measured concentrations of DEHP in single exposure solutions (mg/L)

| Nominal | 0.1 | 0.3 | 0.6 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|---|
| Measured | 0.11 ± 0.01 | 0.28 ± 0.01 | 0.59 ± 0.02 | 0.99 ± 0.01 | 2.03 ± 0.44 | 3.10 ± 0.25 | 3.97 ± 0.54 |
| Nominal | 64 | 96 | 128 | 160 | 200 | 300 | 400 |
| Measured | 64.70 ± 0.61 | 94.66 ± 1.77 | 125.36 ± 0.91 | 158.13 ± 2.19 | 195.21 ± 5.75 | 292.24 ± 5.13 | 386.35 ± 6.77 |

**Table 3** The nominal and measured concentrations of Cu(II) in single exposure solutions (mg/L)

| Nominal | 0.025 | 0.075 | 0.15 | 0.25 | 0.5 | 0.75 | 1 | 4 |
|---|---|---|---|---|---|---|---|---|
| Measured | 0.025 | 0.074 | 0.15 ± 0.01 | 0.24 ± 0.03 | 0.51 ± 0.02 | 0.77 ± 0.04 | 1.06 ± 0.09 | 4.22 ± 0.15 |
| Nominal | 5 | 6 | 8 | 9 | 10 | 11 | | |
| Measured | 5.05 ± 0.57 | 6.03 ± 0.42 | 8.11 ± 0.44 | 9.08 ± 0.56 | 10.05 ± 0.48 | 11.07 ± 0.75 | | |

**Table 4** The nominal and measured concentrations of DBP in mixed exposure solutions (mg/L)

| Nominal | 3.2 | 6.4 | 8 | 9.6 | 12.8 | 97.5 |
|---|---|---|---|---|---|---|
| Measured | 3.17 ± 0.06 | 6.25 ± 0.09 | 8.07 ± 0.03 | 9.58 ± 0.03 | 12.93 ± 0.89 | 95.68 ± 1.11 |
| Nominal | 195 | 243.75 | 292.5 | 341.25 | 390 | |
| Measured | 191.28 ± 2.23 | 237.31 ± 4.87 | 286.63 ± 6.54 | 335.08 ± 6.76 | 384.71 ± 8.63 | |

**Table 5** The nominal and measured concentrations of DEHP in mixed exposure solutions (mg/L)

| Nominal | 0.8 | 1.6 | 2 | 2.4 | 3.2 | 60.4 |
|---|---|---|---|---|---|---|
| Measured | 0.78 ± 0.01 | 1.60 ± 0.01 | 1.98 ± 0.02 | 2.45 ± 0.03 | 3.28 ± 0.04 | 61.27 ± 1.13 |
| Nominal | 120.8 | 181.2 | 241.6 | 302 | 362.4 | |
| Measured | 122.16 ± 2.54 | 182.70 ± 3.61 | 238 ± 3.97 | 299.10 ± 4.91 | 355.68 ± 5.63 | |

**Table 6** The nominal and measured concentrations of Cu(II) in mixed exposure solutions (mg/L)

| Nominal | 0.056 | 0.112 | 0.14 | 0.168 | 0.224 | 1.4 | 1.75 | 2.8 |
|---|---|---|---|---|---|---|---|---|
| Measured | 0.061 | 0.111 ± 0.01 | 0.14 ± 0.01 | 0.17 ± 0.03 | 0.23 ± 0.02 | 1.35 ± 0.15 | 1.92 ± 1.20 | 2.72 ± 0.45 |
| Nominal | 3.5 | 4.2 | 4.375 | 5.25 | 5.6 | 6.125 | 7 | 8.4 |
| Measured | 3.54 ± 0.13 | 4.31 ± 0.59 | 4.39 ± 0.31 | 5.32 ± 0.51 | 5.68 ± 0.24 | 6.18 ± 0.62 | 7.16 ± 0.55 | 8.57 ± 0.64 |

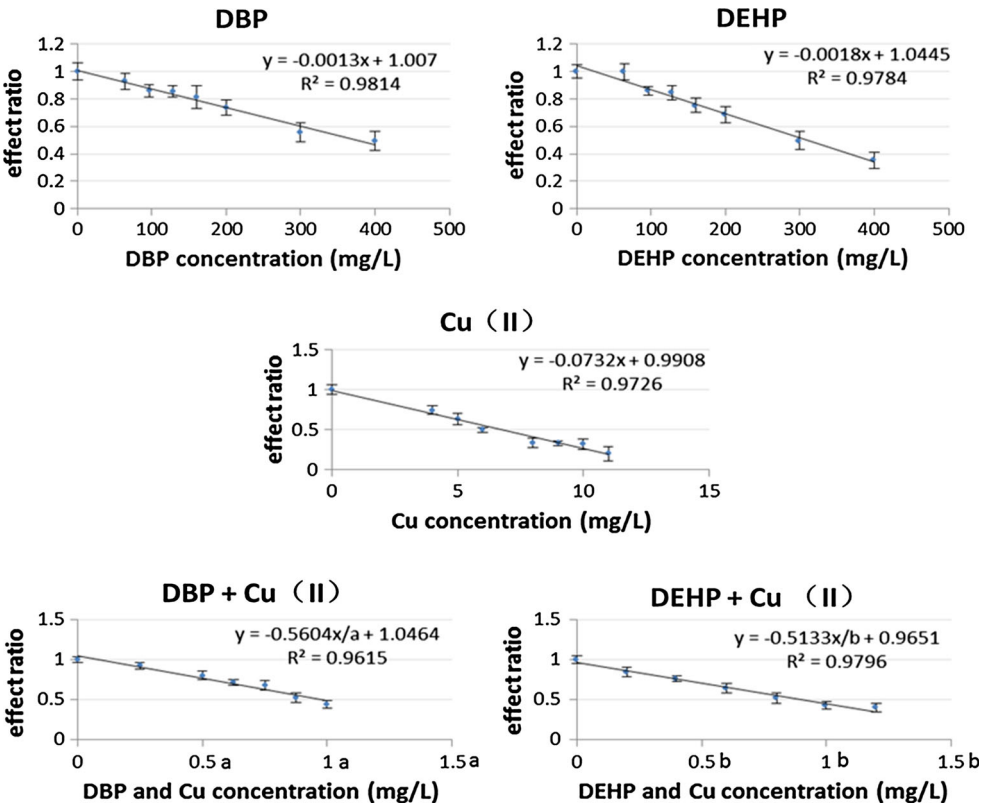


**Fig. 1** Effects of single and joint toxicitiy of DBP, DEHP and Cu(II) to *Photobacterium phosphoreum* in 3 % NaCl solution

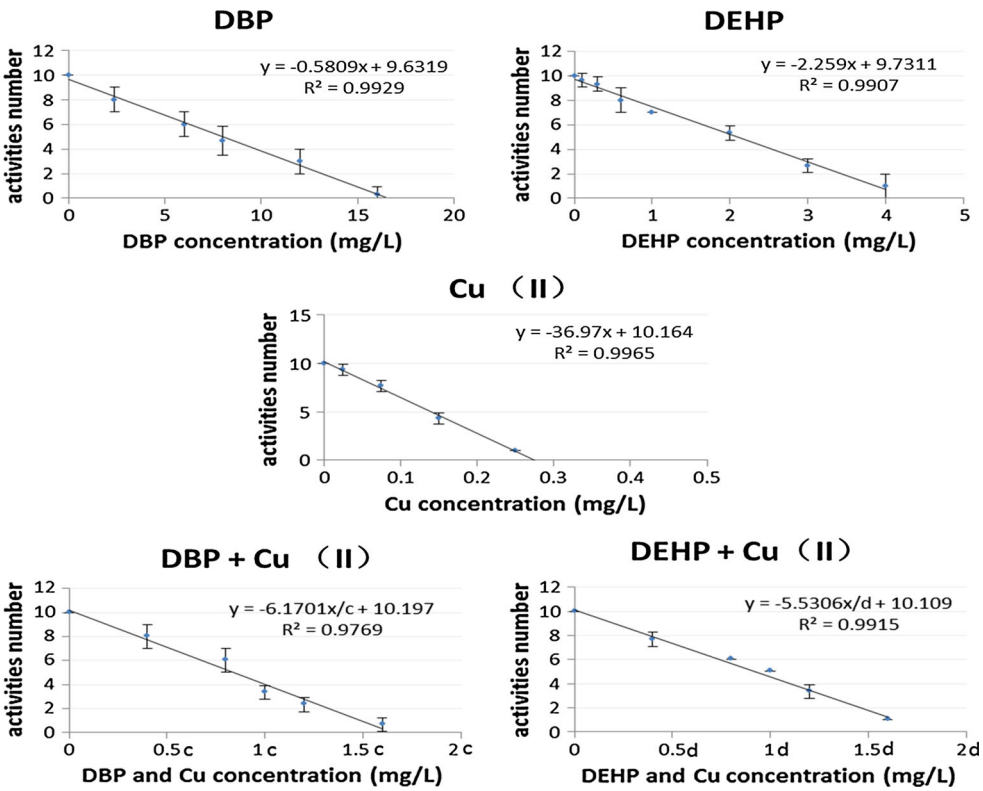


**Fig. 2** Effects of single and joint toxicitiy of DBP, DEHP and Cu(II) to Daphnia magna for 24 h

the tested toxicants. The toxicity sequence of single chemicals to *P. phosphoreum* was Cu(II) > DEHP > DBP, judged by their $EC_{50}$. Some comparative research studies have also found DEHP to be more toxic than DBP toward aquatic organisms (Adams et al. 1995; Zheng et al. 2013). Call et al. (2001) reported increased toxicity of several phthalate esters with increasing log Kow and lipophilicity for phthalate esters from DMP up through BBP. We propose that it may be explained by lipid solubility; in general, a molecule with higher molecular weight and longer carbon chain (i.e. DEHP) has the higher lipid solubility (Small et al. 1947). The effect of joint toxicity was shown as antagonism, which means that the joint toxicity of DBP and DEHP combined with Cu(II) was lower than the sum of their individual toxicities.

The $EC_{50}$ values for *D. magna*, by single chemical or mixtures, were nearly two orders of magnitude lower than that of *P. phosphoreum,* which indicated that *D. magna* was more sensitive to these toxicants. The toxicity sequence was also Cu(II) > DEHP > DBP. When mixed with Cu(II), the toxicity of mixtures was less than the sum of two compounds. So the joint toxicity also showed an antagonism effect to *D. magna*.

Our previous study on the toxicity of DBP, DEHP and Cu(II) to *Carassius auratus* also showed an antagonism effect (Huang et al. 2015). Mehler et al. (2011) researched the joint toxicity of an organic compound, cypermethrin, and a heavy metal, lead, to *Chironomus dilutus*, and found that the joint toxicity type was antagonism.

*P. phosphoreum* and *D. magna* are of significant difference in terms of biological levels, and they represent two different categories of aquatic organisms. The toxicity to *D. magna* was almost two orders of magnitude higher than *P. phosphoreum*. Some earlier studies have also found the two species to be similar to our results (García et al. 2001; Yu et al. 2009). This may be largely due to differences in organism type, as *Daphnia magna* is commonly amongst the more sensitive species to toxicants in aquatic toxicity tests, and bioluminescent bacteria are often amongst the least sensitive. The observed difference in sensitivity may also be due in part to different durations of exposure. It has been proposed that differences in exposure time can cause significant differences in $EC_{50}$ values between different organisms (Deneer et al. 1989). The longer exposure time usually leads to greater toxicity and a lower value of $EC_{50}$. In this experiment, the exposure time of *D. magna* was 24 h, while the exposure of *P. phosphoreum* only lasted for 15 min. Some other factors, such as the individual size and solvent ingredient, may also contribute to the different toxicity between *P. phosphoreum* and *D. magna*.

In conclusion, the toxicity order was Cu(II) > DEHP > DBP for both test organisms. The toxicity of the mixtures of the two PAEs with Cu(II) showed an antagonistic effect, i.e., the joint toxicity was lower than the sum of two compounds. *D. magna* was more sensitive than *P. phosphoreum* when exposed to the toxicants. The results of this experiment are the first to express the joint toxicity of waterborne PAEs with a metal ion on plankton and aquatic bacteria, which could guide a more realistic assessment on the potential toxicity of different pollutants on the aquatic organisms.

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