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The Acute Toxicity of Phenolic Compounds and Heavy Metals on Brine Shrimp *Artemia sinica* (Crustacea: Artemiidae)

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Abstract: The brine shrimp *Artemia* has many advantages as a standardized species for toxicity screening of estuarine and marine conditions, widely used for the evaluation of different environmental contaminants including organic compounds as well as heavy metals. In the present investigation four phenolic compounds and heavy metals were tested against 48-hr old brine shrimp *Artemia sinica*. The toxicity order for phenolic compounds and heavy metals was, n-NP > *t*-OP > 2-4-DCP > BPA and Cu > Cr > Cd > Pb respectively. Thus, *A. sinica* nauplii possess significant resistant to heavy metals as compared to phenolic compounds.

Keywords: *Artemia sinica*, toxicity, phenolic compound, heavy metal

1. INTRODUCTION

Many living organisms have been used as bioindicators to assess the toxicity of heavy metals and other toxic substances to natural ecosystems. Among crustaceans, brine shrimp *Artemia* species are extensively used in research and laboratory bioassays worldwide. There are several reasons for the selection to using *Artemia* as a test organism in hazard assessments without the necessity of maintenance of stocks. *Artemia* has strong adaptability to hyper saline environments (5-250 gl^{-1}), temperature (6-35°C), and a good predictive potential as alternatives for other crustacean tests species and flexibility to varied nutrient resources as it is a non-selective filter feeder (Persoone and Peter, 1987), the continuous availability of *Artemia* in the form of dry cysts (encapsulated embryos), the low cost, substantial amount of relevant literature and the fact that they have small body size, which can be easily stored and shipped to any point of the world (Trieff, 1980; Hadjispyrou *et al.,* 2001).

Many studies have reported the acute toxicity of environmental contaminants on different species of genus *Artemia* (Castritsi-Catharios, 1989; Migliore *et al.,* 1997; Melahat, 1998; Castro-Mejia *et al.,* 2011), however the present study is the first report elucidated *Artemia sinica* as a test organism for short-term acute toxicity while using phenolic compounds and heavy metals. This species of *Artemia* is existed in coastal areas of China and extensively used as a valuable food source for aquatic invertebrates and in aquaculture development. The alkylphenol ethoxylates are commercially important surfactants with industrial, agricultural, and domestic applications during the last several decades with an annual production of 50, 0000 ton, in which nonylphenol ethoxylates is reported about 80% (Fu *et al.,* 2007). It has also been demonstrated that alkyl phenols, especially nonylphenol and octylphenol are more hydrophobic and having endocrine disrupting properties (Ackermann *et al.,* 2002; Xu *et al.,* 2006).

The present study was conducted to investigate the acute toxicity of phenolic compounds n-nonylphenol (n--NP),4-*tert*-octylphenol(4-*t*-OP), 2-4-dichlorophenol (2-4-DCP) and bisphenol-A) and heavy metals (Cu, Cr, Ni, Pb) against 48-hr old *Artemia sinica* to determine the 24-hr LC_{50} (concentration of the toxicant which kills 50% of the test animals after 24-hr exposure).

2. MATERIALS AND METHODS

The brine shrimp *A. sinica* obtained from Bohai Bay Brand (Dongying Ocean Artemia Co. Ltd., China) in encysted eggs dry state were hatched following the procedure of (Barahona-Gomariz *et al.,* 1994), was applied and modified. The dry eggs were hydrated in pre-filtered (0.45 µm) and autoclaved seawater 30 ppt, 7.5 pH and constant temperature at 27°C (using electronically controlled Hopar aquarium heater 230v-50 Hz) throughout the incubation period for 24 hours. It should be remember that the seawater used in the whole experiment was collected from shrimp culture pounds along the Yellow sea, Qingdao, China, and expected little contamination background. The entire process of cysts hatching was carried out in a graduated beaker (2000 ml) with continuous side illumination (20-W fluorescent lamp) and kept in suspension by a gentle aeration stone maintained through a small tube in contact with the bottom of the beaker. To avoid changes in salinity due to evaporation, masking tape was used outside the beaker and covered it accordingly. The neonates produced were aspired with Pasteur pipettes and transferred to a 100 ml glass beaker containing the desired volume of freshly filtered and autoclaved seawater. The beaker then incubated for further 24 hours in 12/12 hour light/dark cycle at 25 ± 1°C, to let the larvae to molt to the second or third instar stages.

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For phenolic compounds (n-Nonylphenol, technical grade) (n-NP), bisphenol A (BPA, 97%), 4-*tert*-octylphenol (4-*t*-OP, 97%) and 2-4-dichlorophenol (2-4-DCP, 99%) were purchased from Sigma-Aldrich. Hexane and acetone at HPLC grade were from Tedia. Four heavy metals Cu ($CuSO_4 . 5H_2O$), Cr (Na_2Cr0_4), Cd ($CdCI_2.2.5H_2O$) were obtained from Fuchen chemical, Tianjin and Pb ($(CHCOO)_2$ -Pb.3H_2O), Shanghai.

A mass of 0.1 g of each standard substances, 4-*t*-OP, n-NP, 2-4-DCP and BPA were taken in a beaker with a small amount of volume ratio of hexane: acetone = 8:2. The mixed solvent then transferred into 100 ml volumetric flask, of constant volume to get a concentration of 1000 mg/l of each single standard stock solution. In order to obtain the desired concentration of heavy metals, one gram (1 g) of each metal was taken in 1000 ml volumetric flask containing distilled water and dissolved to get a concentration of 1000 mg/l of each metal stock solution.

For toxicity testing of phenolic compounds, the method of (Hadjispyrou *et al.*, 2001) was applied and modified. While testing phenolic compounds, multiwell plates (12 well cluster flat bottoms with lid and sterile, Costar 3513, USA) were used in which 10 animals were placed in each wells having 5 ml toxicant solution with salinity of 30 $^0/_{00}$ and 7.5 pH. The pre-filtered (0.45 µm) and autoclaved seawater were used in the entire process. Before the main test, a series of preliminary tests for all toxicants were carried out and each group of animals was dosed to determine the critical range (LC_{50}), and a range of concentration between the highest no-observed-effect level (NOEL) and the lowest observed-effect level (LOEL). After all, the subsequent range of concentrations for each compound was applied and repeated accordingly. Each dose of chemical was tested in triplicate with control wells to monitor the death of the animals and the results were declared valid if the mortality in the control did not exceed beyond 10%. The animal was considered dead if no movement of the appendages is observed within 10-15 seconds while gently touched with a prodder. The proposed phenolic compounds experiment was repeated three times. After the incubation at 25 ± 1°C for 24 h, dead animals were observed and counted using stereomicroscope (Nikon YS100, Japan) for each toxicant concentration and the mean percentage mortality was evaluated.

The heavy metals toxicity experiment was performed using glass beakers. A batch of 10 *A. sinica* was placed in 100 ml beaker containing 20 ml pre filtered and autoclaved seawater and incubated for 24 hours. Several introductory tests were performed to get the desired concentration of the toxicant that would be used in the main test. The condition of the specimens in the beaker was observed at regular interval of 5 h, using stereomicroscope. The animal was declared dead when it is failed to respond any mechanical stimulation. All the experimental conditions including temperature, salinity and pH were the same as used in the prior (phenolic compounds) test. Each metal experiment was repeated five times.

The statistical analysis of both phenolic compounds as well as heavy metals toxicity was carried out by taking into consideration the mean percentage values of the dead animals after 24-hr exposure and the median lethal concentration (LC_{50}) was obtained by linear regression equation.

3. RESULTS AND DISCUSSIONS

The toxicity of four phenolic compounds (n-NP, 4-*t*-OP, 2-4-DCP and BPA) and heavy metals (Cu, Cr, Cd and Pb) against *A. sinica* were investigated. After three introductory tests the following range of concentrations were followed: 0.1, 0.453, 0.454, 0.455 and 0.8 µg/L for n-NP; 0.8, 1.52, 1.53, 154 and 6 µg/L for 4-*t*-OP. The highest NOEL for n-NP was 0.1 and 0.8 µg/L for 4-*t*-OP. The results are demonstrated in figure 1. Figure 2 illustrates the effect of 2-4-DCP and BPA on the mortality of *A. sinica*. After three preliminary tests the following range of concentrations were used for 2-4-DCP: 1, 3.53, 3.56, 3.58 and 10 mg/l while 10, 45.56, 45.59, 46.40 and 90 µg/L for BPA. The highest NOEL between the two compounds was 1 and 10 µg/L for 2-4-DCP and BPA respectively.

The following Table summarizes the median lethal concentrations values (LC_{50}) that caused 50% mortality in *A. sinica*. It can be seen that the toxicity of the phenolic compounds and heavy metals tested in this study was in the order of n-NP > 4-*t*-OP > 2-4-DCP > BPA and Cu > Cr > Cd > Pb respectively. In the current study the toxicity variation between the tested toxicants is one or more orders of magnitude. **(Fig.1and 2).**

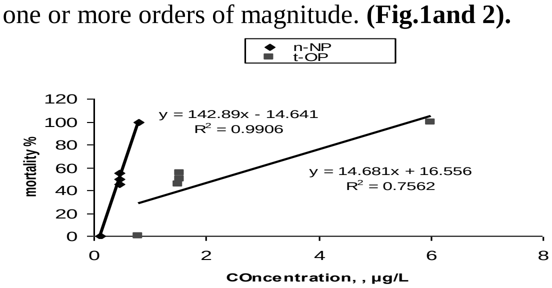

Fig.1. Toxicity of n-NP and 4-t-OP on *A. sinica.*

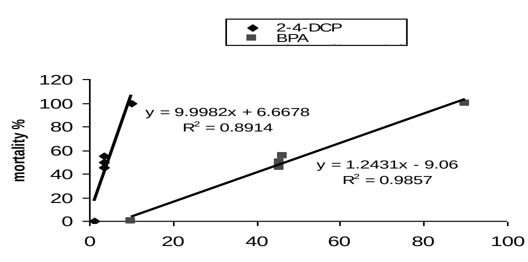

Fig. 2. Toxicity of 2-4-DCP and BPA on *A. sinica*

The brine shrimp *Artemia* has many advantages as a marine test organism and widely used for the evaluation of different types of contaminants including organic and inorganic metals (Hadjispyrou *et al.,* 2001). *Artemia* has also been used as a standardized species for toxicity assessment of estuarine and marine conditions phenolic compounds (Guerra, 2001), water treatment and cyanobacteria blooms (Tothill and Turner, 1996) and the appraisal of harmful toxic effects of composts (originating from industrial waste) on ecosystems (Kapanen and Ita¨vaara, 2001; Nunes *et al.,* 2006). In the present investigation four phenolic compounds were tested against 48-hr old *A. sinica.* Our results are in agreement with other researchers reporting that, *A. salina* larvae aged 48-hr appear to be one of the most vulnerable age to phenolic compounds (Barahona and Sánchez-Fortún, 1996) and some organic solvents and pesticides (Sánchez-Fortún and Barahona, 1995). The toxicities of n-NP and 2-4-octylphenol were obvious among the four phenolic compounds **(Fig.1).** The extant of toxicity in terms of 24-hr LC_{50} values mg/l, n-nonylphenol is about 1.8 times more toxic than LC_{50} of 4-*t*-OP, 3.8 and 47 times than 2-4-DCP and BPA respectively **(Table-1).**

Table 1 . The 24 hr-LC50 values in µg/L for phenolic compounds and heavy metals tested against *A. sinica* obtained by linear regression equation

Test Chemicals	24-h LC_{50} (SD)
n- Nonylphenol	0.45 ± 0.001
4 - *t*- Octylphenol	2.27 ± 0.01
2-4-Dichlorophenol	4.33 ± 0.02
Bisphenol-A	47.51 ± 0.47
$CuSO_4.5H_2O$	33.74 ± 4.95
Na_2Cr0_4	162.11 ± 5.16
$CdCI_2.2.5H_2O$	185.36 ± 2.21
$(CHCOO)_2$ -Pb.$3H_2O$	282.69 ± 4.33

SD: Standard Deviation

It can be seen that there is a significance variations among the toxicities of four phenolic compounds. The toxicity deviation among the phenolic compounds can also reflect the age factor and may be different at different life stages of the tested animal. According to the results of the toxicity of pure pentachlorophenol was equal to all three ages of *Daphnia magna* (young, juvenile and adult) but the toxicity of technical pentachlorophenol decreased with maturation of the same organism and the toxicities of o-nitro phenol and 2, 6-dimethylphenol were 3-6 times more for 48-hr old *Artemia salina* than *A. salina* 24-hr old respectively and therefore supported our results.

For heavy metal toxicity experiment several range finding tests were performed for each metal to get the desired range of concentration. After four range-finding tests the following concentration were tested for Cu: 2.5, 20.4, 25.5, 30.3 and 90 µg/L while 25, 140, 145.22, 150.32 and 350 µg/L for Cr. The highest NOEL for Cu was 2.5 and 25 µg/L for Cr. The results are displayed in

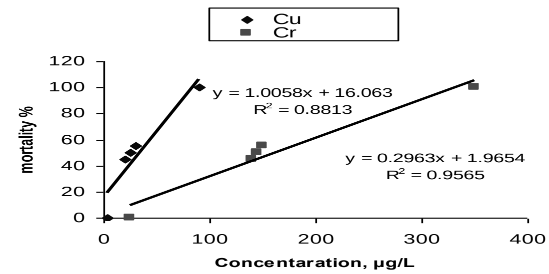

Fig. 3. Toxicity of Cu and Cr on *A. sinica.*

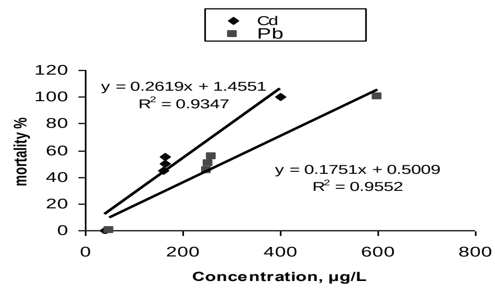

Fig. 4. Toxicity of Cd and Pb on *A. sinica*

(Fig. 3). Similarly, the selected ranges of concentrations for Cd were 40, 160, 162.33, 164.43 and 400 µg/L and 50, 250, 254.53, 258.65 and 600 µg/L for Pb. The highest NOEL between the two metals was 40 and 50 µg/L for Cd and Pb respectively **(Fig. 4).**

Generally, it is believed that all species of genus *Artemia* are not very sensitive to metals and the relative sensitivity with other aquatic organisms range from moderately sensitive to insensitive to a wide range of metals (Brix, *et al.,* 2006). According to our results, the established order of toxicity for four heavy metals against *A. sinica*; Cu > Cr > Cd > Pb. Copper and Chromium are declared to be the most toxic among the four heavy metals. In the present study the toxicity in terms of 24-hr LC_{50} values µg/L, Cu is 128.4 times more toxic than Cr, 151.6 and 248.9 times than Cd and Pb respectively While conducting toxicity experiment for brine shrimp *Artemia*, (Gajbhiye and Hirota, 1990) recognized the following order of tested heavy metals, Pb > Cd > Cu > Ni > Zn > Fe > Mn. Other researches (MacRae and Pandey, 1991) declared that Cu and Pb are about equally toxic, reducing the rate and extent of *Artemia franciscana* development at or below concentrations of 0.1 µM, Zinc was somewhat less toxic than copper and lead, while nickel was the least toxic. Sensitivity studies on the early development and growth of *Artemia franciscana* also reported surprisingly to ambient metal concentrations at significant risk from Cu and Zn in Great Salt Lake , UT, where ambient concentrations as high 10 and 14 µg l^{-1}, respectively. In most of other test organisms for instance, *Daphnia magna* and *Chlorella ellipsoidea* the toxicity of Cd is reported higher than Cr (VI) The toxicity deviation among heavy metals to different species of brine shrimps may be due to their geographical distribution, physiological and biochemical composition as well as the experimental methods pursued. However, our results are consistent with the other researchers work, (Brown, Ahsanullah, 1971 and Hadjispyrou *et al.,* 2001), established the following order of toxicities; Hg > Cu > Cd > Fe > Zn > Pb and Cr > Cd respectively.

It is concluded that the brine shrimp *A. sinica* nauplii possess a significant resistant to heavy metals as compared to phenolic compounds. The toxicity range of the above tested chemicals may be also varied with the life stages of the *A. sinica*. Therefore further investigation is suggested for the assessment of these harmful environmental contaminants using fully developed brine shrimp *Artemia,* a standardized model species for the toxicity screening of estuarine and marine ecosystems.

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